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Cytopathologic alterations induced by the agent of ovine chlamydial polyarthrititis

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Cytopathologic alterations induced by the agent
of ovine chlamydial polyarthrititis

by

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INTRODUCTION

Ovine chlamydial polyarthrititis (OCP) is one of a number of arthritides affecting lambs. Recognition and description of the disease and its cause occurred within the last decade (Mendlowski and Segre, 1960; Mendlowski et al., 1960; Storz et al., 1963; Shupe and Storz, 1964; Livingston et al., 1965; Pierson, 1967). Earlier recognition of the causal agent was undoubtedly hampered by the technical difficulties encountered in isolation and identification of agents of the genus Chlamydia (Psittacosis-lymphogranuloma agents). Ovine chlamydial polyarthrititis is an acute febrile disease of lambs which occurs in epizootic proportions in the western and southwestern United States. That found in other states has been limited to lambs originating in the West. It is characterized clinically by fever, listlessness, conjunctivitis, and severe lameness which may meliorate in 2-3 weeks or become chronic. Descriptions of pathologic changes differ somewhat but basically consist of inflammation of synovial and subsynovial structures of most freely movable joints of the limbs. In chronic cases, there is fibrous thickening of the periarticular structures. A systematic study of the cytopathic effects of this agent by either light or electron microscopy has not been described, nor have the ultrastructure or replication of the causal agent been defined.

This study is an extension of the first reported outbreak

of OCP in Iowa (Page and Cutlip, 1968). In the fall of 1966, a flock of feedlot lambs in northern Iowa developed signs and lesions typical of those reported for OCP. An organism was isolated in embryonated chicken eggs from affected joints and the disease experimentally reproduced. The agent was identified as a member of the genus Chlamydia by characteristic growth in embryonated eggs and by antigenic analysis. It was further characterized as belonging to the species Chlamydia psittaci by resistance to the growth inhibitive effects of sodium sulfadiazine and failure to produce iodine-stainable carbohydrates during growth. Difficulties encountered during this outbreak in attempting to demonstrate the agent in tissues by histopathologic and electron microscopic techniques and the aforementioned undetermined factors of the disease prompted additional studies.

This project was designed to determine the ultrastructure and replicative cycle of the OCP agent, to further evaluate pathogenesis and cytopathology of the articular lesion, and to appraise microscopic methods of detecting the organism. Toward these ends, several techniques were employed, including histopathologic, histochemic, immunohistochemic, and electron microscopic methods on infected cell cultures and ovine synovial tissues.

REVIEW OF LITERATURE

Ovine Chlamydial Polyarthrititis

Ovine chlamydial polyarthrititis was described for the first time as a distinct entity in western lambs shipped to a Wisconsin feedlot during the fall and winter of 2 successive years, 1957 and 1958, (Mendlowski and Segre, 1960; Mendlowski et al., 1960). There were 3% and 7% mortalities in the respective years. The disease was characterized by an insidious onset with the highest losses approximately one month after the lambs were received. The most notable sign was lameness with a particular rigidity of the affected limbs. Fever, depression, loss of weight, and anorexia were constant, but complete anorexia occurred only in the most severely affected lambs. Most lambs eventually recovered but a few were permanently lame. Their major necropsy findings were serous to fibrinous synovitis with periarticular edema and petechiation. Microscopically, the synovium was edematous, infiltrated with mononuclear cells and overlaid with a fibrinocellular exudate. Synovial intimal cells were swollen and in various stages of degeneration. Changes were not observed in the articular cartilages or in adjacent bones. Numerous minute coccoid bodies, which they interpreted as chlamydial elementary bodies, were seen with Giemsa and Macchiavello stains in synovial inflammatory cells and free in the exudate of the joints. Minor inflamma-

tory changes were observed in the liver and lungs. They cultivated the chlamydial agent in yolk sacs of chicken embryos and experimentally reproduced the disease. Guinea pigs were shown to be susceptible; whereas, mice were resistant to experimental infection. Articular lesions in experimentally infected lambs were the same as in naturally infected lambs; however, changes in other tissues were more extensive. There were interstitial pneumonia, hyperplasia of the reticular cells of the spleen, proliferation of Kupffer cells, and degeneration of hepatocytes and renal epithelial cells. With Giemsa and Macchiavello stains, they found elementary bodies in impression smears and sections of these parenchymal organs as well as in the articular tissues and exudate.

Storz et al. (1963) and Shupe and Storz (1964) reported the disease, known locally as "stiff lamb disease," to be widespread among range lambs in the intermountain region of the West. Most lambs affected weighed from 55-105 pounds; larger lambs were more frequently involved than smaller ones. Morbidity varied among flocks from 2-75%. Mortality was reported as low. Penicillin was effective during the acute stage of the disease but later was ineffective. Such chronically affected lambs did not recover to make satisfactory weight gains. Signs and lesions were similar to those described by Mendlowski and Segre (1960). However, Shupe and Storz (1964) reported changes in the articular cartilage of some lambs. These were slight erosion of the surface of

articular cartilages and marginal compensatory changes (lip-ping). The round ligament of the coxofemoral joint was shredded and fibrillated in some lambs. They also found meningoencephalitis, characterized by gliosis, focal accumulations of inflammatory cells and mild vasculitis. Intracellular elementary bodies were found in smears of the choroid plexus. They characterized the disease as a "polyarthrititis, tendovaginitis (tendosynovitis), tendofascitis and periarticular myositis" and stated the "most striking change appeared to be articular and periarticular reactive fibroblast-like proliferation." The synovial changes were considered to be a "granulomatous, inflammatory, hyperplastic reaction."

The pathogenesis of chlamydial polyarthrititis was studied by Storz et al. (1965) in experimentally infected lambs. Lambs were exposed by either intravenous, intramuscular, or intraarticular injection of chicken-egg-yolk-sac-propagated agent and killed 3, 7, and 21 days postinoculation. Intra-articularly inoculated lambs were lame in those joints exposed directly 12 hours postexposure and manifested lameness in other limbs 6 days after inoculation. The intravenously and intramuscularly inoculated lambs developed stiffness 3 days after inoculation and were obviously lame within 5 days of exposure. The authors were able to reisolate the agent from all lambs (joints and various internal organs) at each of the 3 termination periods, regardless of the route of administration. Thus, the agent did not remain localized in the joints

of lambs inoculated intraarticularly. Articular lesions were minimal (increased fluid, petechiae and hyperemia) at 3 days postexposure. Lesions were extensive and similar, 7 and 21 days after inoculation, regardless of the route. They found an increased amount of fibrin-containing synovial fluid, hyperemia and petechiation of the synovial membranes and tendon sheaths, swollen synovial cells of irregular size, and accumulations of mononuclear cells in the subsynovial tissues which were regarded as granulomatous in nature. Polymorphonuclear cells were scattered among the synovial cells. The kidneys, hearts, livers, lungs, lymph nodes and ependymal tissues were hyperemic, edematous, and infiltrated with lymphocytes and monocytes.

In 1965, Livingston et al. reported the disease to have been enzootic in sheep raising areas of Texas for 15 years. Lambs 2-4 months of age were affected most frequently with an average morbidity of 80% in infected flocks. Deaths were infrequent. Complement fixation tests were considered useful as a diagnostic aid in older lambs but not in young lambs which were believed to be incapable of producing detectable complement fixing antibodies.

Pierson (1967) studied OCP in a large number of feeder lambs in Colorado. He stated the disease had probably existed in Colorado for 25 years and that it was one of the most common nonfatal diseases of feedlot lambs in that state. The average morbidity rate of epizootics involving 80,000 lambs since 1951

was less than 10%, but in some feedlots approached 100%. Most outbreaks were recorded during October and November, 2-4 weeks after transporting the lambs. Signs and lesions were similar to those reported by others. Tylosin, oxytetracycline, chlorotetracycline, and penicillin were reported to be effective therapeutic drugs when used early in the course of the disease. He considered tylosin and oxytetracycline to be the most efficacious of the antibiotics tested.

An outbreak of OCP in Iowa (Page and Cutlip, 1968) involved 2% of a flock of 2,000 feeder lambs which had originated in Idaho and South Dakota. A tentative diagnosis was based on signs, lesions and serologic evidence and confirmed by isolation of the organisms in embryonated chicken eggs. The disease was reproduced in 3-month old lambs by the intravenous and intraarticular routes.

In addition to confirmed reports of OCP, Shupe and Storz (1964) suspected chlamydiae as the cause of lameness in lambs reported by Griner (1959) and by Biberstein et al. (1959). Griner (1959) described a number of outbreaks of polyarthrititis of unknown etiology in feeder lambs in Colorado. The lambs had been shipped to Colorado feedlots from several western and southwestern states including Wyoming, Montana, Colorado, Texas, and New Mexico. Lameness appeared 3-4 weeks after shipment. He described a fibrinopurulent polyarthrititis characterized by thickened, edematous synovial membranes containing fibrin, neutrophils, lymphocytes, and macrophages, with

fibroblastic proliferation along the luminal surface, myositis at the myotendon junction and an increased volume of synovial fluid containing fibrin and neutrophils. He attempted to transmit the disease to mice, guinea pigs, and normal lambs with synovial fluid from arthritic lambs but was unsuccessful. Biberstein et al. (1959) reported a similar disease of unknown cause in nursing and feeder lambs in California.

Marsh (1947) reported a nonsuppurative arthritis of lambs in several areas of the western United States from which Corynebacterium ovis was isolated and believed to be the cause. He later (Marsh, 1965) suggested this may have been OCP.

The mode of transmission of the OCP agent is uncertain but has been assumed to be via inhalation or consumption of infectious particles shed in feces or urine. Separate chlamydial isolates from the feces and joints of arthritic lambs have been shown by serum neutralization to be antigenically similar (Storz, 1966). Conjunctival infection has also been proposed as a route of entry for the polyarthritis agent (Storz et al., 1967).

Structure and Replication of Chlamydial Agents

The mode of replication of members of the genus Chlamydia has been studied for more than 60 years. As early as 1907, Halberstaedler and von Prowazek, as cited by Weiss (1949, p. 125), observed intracytoplasmic inclusions of the trachoma agent in conjunctival smears stained with Giemsa. They de-

scribed blue inclusions containing red granules and believed the red granules represented the trachoma agent and the blue matrix was of cellular origin. Linder, in 1909 and 1910, as mentioned by Weiss (1949, p. 125), observed that trachoma inclusions in tissue sections were composed of intracytoplasmic granules of different size and staining characteristics; the larger blue granules he termed "initial bodies" and concluded they divided and eventually formed smaller red granules or elementary bodies.

The first systematic investigation of the growth cycle of a member of the genus Chlamydia was done by Bedson and Bland in 1932. Similar investigations using light microscopy and conventional staining of infected tissues and cell cultures have been done with various chlamydiae, including the agents of psittacosis (Bedson, 1933; Bedson and Bland, 1934; Bland and Canti, 1935; Levinthal, 1935; Yanamura and Meyer, 1941; Bedson and Gostling, 1954; Officer and Brown, 1960), trachoma (Gordon et al., 1960), lymphogranuloma venereum (Rake and Jones, 1942), meningopneumonitis (Pinkerton and Moragues, 1942), mouse pneumonitis (Karr, 1943), and ovine enzootic abortion (Stamp, 1951). Except for minor variations, these authors agreed that elementary bodies developed within the cytoplasm of cells into larger initial bodies after a latent period of 5-20 hours. The initial body was considered the vegetative form and division was regarded to be by repeated binary fission, thus giving rise to new ele-

mentary bodies. Lysis of the cell was believed to release the newly formed elementary bodies.

Weiss (1949) claimed extracellular growth of the agents of mouse pneumonitis, feline pneumonitis, and meningopneumonitis occurred in the lungs of mice. The same agents grew only within the cell cytoplasm in embryonated eggs.

A principal difference existed over the nature of the intracytoplasmic inclusion formed. Inclusions were described as dense plaques or diffuse vesicular structures. In their initial report, Bedson and Bland (1932) described a dense inclusion which they called a "plaque" and considered it a true viral matrix; but, they later (Bedson and Bland, 1934) regarded it as closely packed elementary bodies. Some (Bland and Canti, 1935) believed the plaque essential for development of the agents while others (Levinthal, 1935; Yanamura and Meyer, 1941) regarded it as unnecessary. Levinthal (1935) considered the type of inclusion formed related to the degree of resistance offered by the cell. He believed compact inclusions represented comparatively greater cellular resistance. More recent work comparing the growth of various chlamydial agents has shown the density of the inclusion to depend on the particular host-agent system involved (Officer and Brown, 1960; Weiss (1955) stated that the environment influenced the type of inclusion formed; however, some of the strain characteristics of the agent were maintained regardless of the host cell type.

The resolving power of the light microscope was not adequate to settle the question of the appearance of the agents during the latent period (lag phase); i.e., from the time of inoculation until initial bodies appeared in the cell cytoplasm approximately 20 hours later. Many investigators have found a reduced infectivity during the lag phase of the growth cycle (Bedson and Bland, 1932 and 1934; Bland and Canti, 1935; Bedson and Gostling, 1954; Litwin, 1959). Bedson and coworkers were unable to demonstrate the psittacosis agent during the lag phase which they attributed to the size of the agent and limited resolving power of the light microscope. They believed the reduced infectivity of the psittacosis agent resulted during the change-over of the small elementary body form to the large initial body form within the cell. Bedson and Gostling (1954) demonstrated that suspensions with a high content of large forms were less virulent than preparations containing small forms.

Sigel et al. (1951) and Girardi et al. (1952) interpreted the rapid decline of infectivity of meningopneumonitis in chorioallantoic ectoderm of chicken embryos as a noninfectious phase of development. They suggested a true viral development and compared it with that of a bacteriophage. Extensive cytochemical investigations into the growth cycle of the psittacosis and trachoma agents were conducted by Pollard and coworkers (Pollard and Starr, 1962; Pollard and Tanami, 1962; Pollard et al., 1960). Infected cell cultures were studied

with the light microscope after acridine orange and immunofluorescent staining. The findings were correlated with those obtained after treatment of infected cells with various antimetabolites. They interpreted results of these studies as direct proof of a true viral eclipse during the lag phase of the growth cycle and synthesis by the cell of components of the agent.

Observations with the light microscope and one-step growth experiments left many questions unanswered about the developmental cycle of chlamydiae (Armstrong et al., 1963). Of particular interest was the nature of the agents during the lag phase in which the infectivity had repeatedly been shown to be decreased; the significance of which was controversial (Armstrong and Reed, 1964). With the advent of the electron microscope, the means became available by which discrete structure of the agents during the entire developmental cycle could be determined. However, early ultrastructural studies were confusing because of artifacts produced during the processing of tissues.

For technical reasons, the first use of the electron microscope for studying chlamydiae was limited to examination of air-dried or freeze-dried preparations shadowed with heavy metals. Little concrete information was added to the knowledge of the reproductive cycle gained by light microscopy. Air-dried preparations of the agents of psittacosis (Swain, 1955; Heinmets and Golub, 1948; Rake et al., 1946), trachoma

(Collier and Sowa, 1958; Ito et al., 1951), feline pneumonitis (Moulder and Weiss, 1951), and murine pneumonitis (Gogolak, 1953) revealed their mutual pleomorphism. Particles from 500 to 1000 nm in diameter were described. Most of the largest particles consisted of a central electron-dense elevation surrounded by a flat periphery of less density, assumed to be membrane. Smaller particles appeared as rough-surfaced spheres. Elongated particles with central constrictions were occasionally seen. Freeze-drying revealed the flat periphery and rough surface to be artifacts produced by drying in air and established the spherical nature of the meningopneumonitis agent (Crocker and Williams, 1955). Ito et al. (1951) and Swain (1955) interpreted the difference in size and shape of particles of the trachoma and psittacosis agents to represent different stages of development and concluded fission was the basic mode of replication. Heinmets and Golub (1948) studied silica replicas of psittacosis-infected chorioallantoic membranes of chicks and demonstrated entrance of the agent into cells and intracellular particles which suggested division by fission. However, they stated this did not exclude the possibility of incorporation of the agent into normal cellular constituents which then produced new infectious particles.

Thin section electron microscopy has been used by numerous investigators to study the ultrastructure and developmental cycle of different chlamydiae, including the agents of meningopneumonitis (Gaylord, 1954; Tajima et al., 1957;

Litwin et al., 1961; Higashi et al., 1962), psittacosis (Tajima et al., 1957; Litwin et al., 1961; Kajima et al., 1964; Higashi, 1965), feline pneumonitis (Litwin, 1959), trachoma (Mitsui and Suzuki, 1956; Mitsui et al., 1957, 1958, 1962 and 1964; Litwin, 1962; Bernkopf et al., 1962; Armstrong et al., 1963; Higashi, 1965), lymphogranuloma venereum (Armstrong and Reed, 1964), and inclusion conjunctivitis (Mitsui et al., 1967). Structure at different stages during the growth cycle was easily determined; however, elucidation of the structure of transitional forms proved more difficult.

Two structurally distinct types of particles have been described; each type of particle was regarded as indistinguishable regardless of the chlamydial agent examined (Litwin et al., 1961; Higashi, 1964, 1965). The smaller of the two particles (small particle) was 200-300 nm in diameter and corresponded to the elementary body seen by light microscopy. It consisted of a central dense area, generally regarded as the nucleoid, and a peripheral area of less density. Some authors (Mitsui et al., 1964) reported an eccentric nucleoid. The larger of the two particles (large particle) was 500-1000 nm in diameter and corresponded to the initial body seen with the light microscope. It was much less dense than the small particle and had a uniform internal structure of fine granules and filamentous strands. Similar filaments have been observed in the nucleoid of small particles and are believed to represent strands of deoxyribonucleic acid (Armstrong et al., 1963;

Mitsui et al., 1964; Erlandson and Allen, 1964). Intermediate size particles (intermediate particle) have been described; their structure is similar to the large particle except they are smaller and contain a small central nucleoid (Mitsui et al., 1964; Kajima et al., 1964; Anderson et al., 1965). There is general agreement that large particles are most numerous approximately 20 hours after infection; intermediate particles then become apparent and finally at termination of the cycle (30-72 hours after infection), small particles predominate.

Most electron microscopists have reported a single limiting membrane surrounding the various size particles; others (Mitsui et al., 1962, 1964; Gaylord, 1954; Anderson et al., 1965) have found two membranes investing the large and intermediate particles. Armstrong and Reed (1964) considered the two membranes investing the large particles reminiscent of the combined cell wall and cytoplasmic membranes of bacterial cells. Internal membranes have been found in large particles by several investigators, most of whom have attributed their presence to various forms of replication including endosporulation (Gaylord, 1954; Bernkopf et al., 1962; Higashi et al., 1962). Other membranous-like structures ("whorls"), found primarily in large particles, are believed similar to mesosomes of bacteria (Erlandson and Allen, 1964).

Chlamydial replication was first examined in ultrathin sections by Gaylord in 1954. Large and small particles of the meningopneumonitis agent were described; some large par-

ticles had constricted centers and others had multiple dense internal granules. The different particle configurations were considered to signify replication by binary fission and multiple endosporulation. Small particles were believed to represent a spore-like stage. An amorphous substance found within the infected cells was considered of cellular origin and not directly related to reproduction of the agent. Bernkopf et al. (1962) also observed structures within large particles of the trachoma agent which they interpreted to represent a form of endosporulation.

Mitsui and coworkers (Mitsui and Suzuki, 1956; Mitsui et al., 1958, 1962) examined the trachoma agent and concluded replication proceeded by formation of polygonal structures from an amorphous matrix. These polygonal structures and other large particles were thought to divide by "polygonal fission," "sporogenous fission," or binary fission. Also, they found structural evidence suggesting the "possibility that the early forms of the agents are formed from mitochondria."

In 1957, Tajima et al. studied the growth of psittacosis and meningopneumonitis agents and concluded reproduction was of a viral nature. Following a complete morphologic eclipse, they found large amorphous nonmembrane-bound inclusions within the cytoplasm of infected cells. These inclusions, similar to those described by Mitsui et al. (1958, 1962), were regarded as viral precursor matrices. Membranes progressively

enclosed portions of the matrices and thus formed large particles which divided into equal or unequal parts by constriction of their limiting membrane. Small particles were believed to be formed by condensation of large particles.

Following the viral-type reproduction proposal by Tajima et al. (1957), Litwin and coworkers (Litwin, 1959, 1962; Litwin et al., 1961) reported finding no morphologic eclipse or viral matrix in cells infected with the agents of feline pneumonitis, psittacosis, meningopneumonitis, or trachoma. Instead, they found large particles of all the agents studied which indicated a fission process. They were unable to determine if the fission was equal or unequal and suggested the process may be too rudimentary to classify.

Entrance of chlamydial particles into cells was not observed in thin sections until 1962 when Higashi et al. demonstrated phagocytosis of the meningopneumonitis agent by strain L cells. The remainder of the growth cycle was considered similar to that reported by Tajima et al. (1957) except large particles were found not to divide after formation from a viral matrix.

In 1963 and 1964 three additional studies were reported (Armstrong et al., 1963; Kajima et al., 1964; Mitsui et al., 1964) which supported the observations of Tajima et al. (1957) and Higashi et al. (1962). The agents of trachoma and psittacosis were found to become unidentifiable subsequent to phagocytosis by cells. Approximately 20 hours later, reticulated

viral precursor was seen from which large particles were formed. The authors believed that after repeated fission, the large particles condensed to form small particles. Armstrong et al. (1963) found evidence, later supported by Anderson et al. (1965) that small particles were also capable of dividing.

After additional studies, 3 investigators refuted their earlier hypothesis of a viral type replication (Armstrong and Reed, 1964; Higashi, 1964, 1965; Mitsui et al., 1967). They concluded that a morphologic eclipse did not occur and that division was entirely by binary fission after enlargement of the phagocytized particle to the large particle stage. The fission process was believed comparable to the "pinching off" of gram-negative bacteria (Armstrong and Reed, 1964). The amorphous substance, originally believed to be viral precursor, was shown to be an artifact resulting from rupture of large particles which had been damaged by the fixatives (osmium tetroxide and potassium permanganate) and by polymerization of the embedment (merthacrylate monomers) (Armstrong and Reed, 1964; Higashi, 1964).

Electron microscopic detection of the OCP agent in lamb synovial tissues was compared with its isolation in chicken embryos by Norton and Storz (1967). Correlation was excellent. The agent was reported to be recognizable throughout the synovial membrane 7 and 14 days postinoculation. Isolated particles were found in synovial lining cells, in endothelial

cells, and in fibroblasts; numerous mononuclear cells contained inclusions filled with mature particles. After 36 days, the organism could not be demonstrated in synovial tissues by either culture or electron microscopy.

Structure and Function of the Synovial Membrane

Hamerman and Schubert (1962) listed the components of the synovial or diarthrodial joint as the capsule and synovial membrane, ligaments, fibrocartilages, synovial fluid, articular cartilages, subchondral bones and occasionally tendons. According to these authors, the synovial membrane is the glistening, white inner surface of the joint capsule and lines the joint cavity except over the articular cartilages. They reported a gradual transition from the thick bundles of collagen and large blood vessels of the capsule into a more delicate areolar tissue of the synovial membrane, with its fine collagen fibers, capillaries and many cells. The cells on the inner surface of the joint capsule were called synovial cells, intimal cells, lining cells, and surface cells.

Key, as quoted by Ham (1965, p. 470), distinguished 3 morphologic variations of the synovial membrane, based on whether the inner lining cells of the joint capsule rested directly on the fibrous capsule or was separated from it by a layer of areolar or adipose tissue. He considered the surface cells to be grouped close together in the areolar and adipose membranes and widely separated from one another

in the fibrous membrane. In sections stained with hematoxylin and eosin, surface cells of the fibrous membrane were difficult to distinguish from subjacent fibroblasts.

Hamerman et al. (1961) found the tetrazolium technique for demonstrating oxidative enzymes particularly useful in revealing morphology of the lining cells. With this procedure, they found cytoplasmic processes which anastomosed at the surface of the synovial membrane to form a limiting margin.

The synovial lining cells, in contrast to subjacent cells of the synovium and joint capsule, were reported (Hamerman et al., 1961) to stain intensely for lactic dehydrogenase, reduced diphosphopyridine nucleotide diaphorase and reduced triphosphopyridine nucleotide diaphorase and with less intensity for succinic dehydrogenase, isocitric dehydrogenase and acid phosphatase. Glucose-6-phosphate dehydrogenase activity was not found in the lining cells.

Ultrastructure of the normal synovial membrane has been studied in man (Lever and Ford, 1958; Barland et al., 1962; Coulter, 1962), the rabbit (Lever and Ford, 1958; Ghadially and Roy, 1966; Adam, 1966), the pig (Roberts et al., 1969), the rat (Roy and Ghadially, 1967), the calf (Langer and Huth, 1960), the guinea pig (Langer and Huth, 1960; Wyllie et al., 1964), the dog (Langer and Huth, 1960), the cat (Lever and Ford, 1958) and the chicken (Luckenbill and Cohen, 1967).

Barland et al. (1962) described 2 cell types (or 2 states

of activity of a single cell type) in the synovial lining based on their cytoplasmic contents. Type A cell was more numerous and contained a prominent Golgi apparatus, numerous single-membrane-bound vacuoles ($0.4-1.5\ \mu$ in diameter) containing varying amounts of a dense granular material, many filopodia (finger-like surface projections), mitochondria, intracellular fibrils, and micropinocytotic-like vesicles. Type B cell contained large amounts of granular endoplasmic reticulum, fewer large vacuoles, micropinocytotic-like vesicles and mitochondria. They found long cytoplasmic processes of these cells which were usually directed toward the membrane surface where they overlapped and intertwined. The lining cells formed a discontinuous outer margin, 2-3 cells in depth, and gradually merged with the underlying connective tissue where fewer cells, with typical appearance of fibroblasts, were found. The intercellular matrix of the lining cells was an amorphous material similar to that observed in cytoplasmic vacuoles of the type A cell. They found numerous thin nonperiodic filaments in the intercellular matrix but no collagen fibers were present. Langer and Huth (1960) found a distinct basement membrane separating the lining cells from the underlying collagenous tissue in calves, guinea pigs and dogs. In contrast, Coulter (1962) and Roberts et al. (1969) demonstrated fibers of collagen extending to the synovial surface in man and swine respectively. Roy and Ghadially (1967) stated that "in contrast to the cells of the synovial

membrane of other species, the cells of rat synovium form a more or less continuous layer facing the joint cavity. The adjacent cell walls show many invaginations and also structures resembling tight junctions and desmosomes."

Roberts et al. (1969) described 4 types of cells within the synovial membrane of swine. They found mast cells characterized by large (134 nm by 400 nm) irregular shaped cytoplasmic granules, cells with regular outline and well developed granular endoplasmic reticulum, cells with a regular outline and a minimal number of cytoplasmic organelles, and cells with an irregular outline and numerous large vacuoles and pinocytotic vesicles. Acid phosphatase activity was found in the large vacuoles of the latter type cells. Mast cells were reported (Asboe-Hansen, 1950) to be common in the synovium of man and were considered as a possible source of hyaluronate.

Luckenbill and Cohen (1967) examined the synovial membrane of the chicken and found cells similar to those reported by previous workers. They regarded the lining cells as a single type; variation in the organelle content was believed to be a function of their distribution within the cytoplasm and plane of the section.

In 1929, Key, as cited by Ball et al. (1964) observed that synovial lining cells ingested particulate matter injected into the synovial cavity. The next year, Franceschini, as cited by Adam (1966), suggested that synovial membranes

should be considered part of the reticuloendothelial system. The high acid phosphatase activity of the lining cells was interpreted by Hamerman et al. (1961) as evidence for abundant lysosomes, thus indicating these cells may absorb and hydrolyze material in synovial fluid. This view was supported by a suggestion by Barland et al. (1962), based on ultrastructure, that the type A synovial cell may have phagocytic properties.

Ball et al. (1964) found when iron dextran was injected into the joints of rabbits, the vacuolar (type A) cells rapidly took up the iron. Iron was rarely found in the endoplasmic reticulum-containing (type B) cells. They found the iron eventually was concentrated in macrophages just beneath the lining layer of cells and suggested that the macrophages may have arisen from vacuolar lining cells. On the other hand, Adam (1966) found intraarticularly injected colloidal carbon was removed from the synovial cavity primarily by invading macrophages. He stated that these cells could be regarded as synovial cells only in the sense of location. The type A and B cells of the synovium were found to ingest very little of the carbon particles. He suggested that the type B cell, with its extensive Golgi apparatus, was important in the production of mucopolysaccharide.

Luckenbill and Cohen (1967) studied the phagocytic properties of synovial lining cells of the chicken and found that a colloidal suspension of carbon injected into the joint cavity was removed by synovial cells which exhibited both vacuoles

and synthetic organelles such as endoplasmic reticulum and Golgi apparatus. Carbon was found only in surface cells in the early stages (15 minutes and 2 hours) and in later stages (3 and 7 days) deeper within the synovial membrane. They interpreted this as evidence that the synoviocyte removed material from the joint fluid and transported it to the sub-synovial region.

Hyaluronate has been shown to be synthesized by synovial membrane slices (Yielding et al., 1957) and by synovial membrane cell cultures (Castor, 1957; Hedberg and Moritz, 1958) but the exact cell involved was uncertain. The finding of a higher level of oxidative enzymes in the lining cells than in the other connective tissue cells of the synovial membrane, the location of the lining cells on the outermost part of the synovial membrane, and the presence in some synovial membranes of a hyaluronidase-susceptible, metachromatically staining substance in apparently clear spaces enclosed by lining cell processes, led to the suggestion by Hamerman et al. (1961) that the lining cells constitute the major site of synthesis of hyaluronate.

Wyllie et al. (1964) described 2 types of microbodies associated with the large Golgi apparatus of the type A lining cell. They hypothesized that these microbodies represented a storage form of hyaluronate synthesized by the cell. They further suggested that the type B cell may be involved in protein synthesis and secretion. Barland et al. (1962) had

also associated granular endoplasmic reticulum with protein synthesis but said it remained to be determined if type B cells synthesize and secrete protein. Previous to this speculation of protein synthesis by synovial cells, protein of synovial fluid was assumed to originate by diffusion from the plasma (Schmid and MacNair, 1958). Sandson and Hamerman (1962) reported isolating a hyaluronateprotein complex from normal human synovial fluid. Protein accounted for approximately 2% of the complex. The function and origin of the protein were not known but they suggested it may participate in synthesis of hyaluronate by synovial cells or could be added at the cell membrane and play a role in the passage of hyaluronate to the synovial fluid. They (Hamerman and Sandson, 1963) found hyaluronateprotein isolated from human joints with rheumatoid arthritis or gout contained about 10% protein and later (Sandson and Hamerman, 1964) demonstrated by immunoelectrophoresis that the protein of the complex was an alpha-globulin. Blau et al. (1965) have shown by immunofluorescence that hyaluronateprotein is localized in the lining cells and their intercellular spaces. They concluded that the protein moiety of hyaluronateprotein is not a serum protein, but is probably synthesized with the hyaluronate in synovial lining cells. Williamson et al. (1966) found with immunoelectrophoresis that a protein of alpha-globulin mobility, antigenically related to serum alpha₂-macroglobulin and other proteins antigenically related to serum proteins were syn-

thesized by cultured synovial cells.

Barland et al. (1962) summarized the function of the synovial membrane as follows: "It appears likely that the lining cells are the major source of hyaluronate, that they play a role in the turnover of the components of synovial fluid, and that their secretory products in the matrix influence the passage of molecules between blood and joint cavity."

MATERIALS AND METHODS

A general outline of the procedures followed during this study is presented in Table 1.

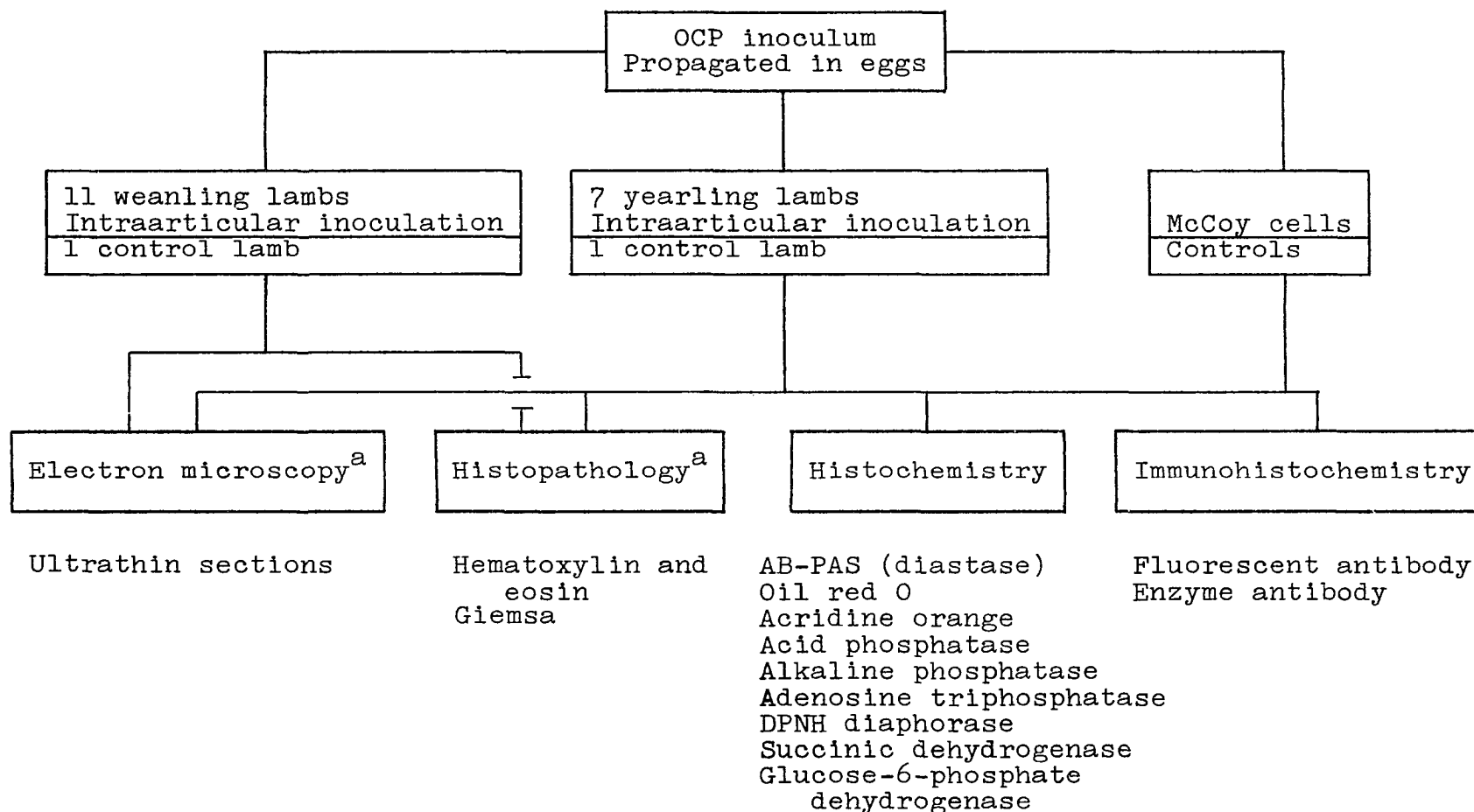
Agent

The organism was isolated from joint exudate of a lamb affected with OCP and identified as a strain of Chlamydia psittaci (Page and Cutlip, 1968). An inoculum of the OCP agent (OCP inoculum) for exposure of cell cultures and lambs was grown in yolk sacs of 7-day chicken embryos. Yolk sacs of embryos dying between 6 to 8 days after inoculation were harvested, homogenized in equal amounts of brain-heart infusion broth (Virtis "45" Homogenizer, The Virtis Company, Inc., Gardiner, New York), and centrifuged in the cold (4°C) for 10 minutes at 3,800 X g. The supernatant was decanted and centrifuged for 20 minutes at 27,600 X g and the sediment thus formed was resuspended in BHI broth and stored at -50°C until used. The chicken embryo LD_{50} of this material after freezing was 0.2 ml of a $10^{-5.6}$ dilution (Reed and Muench, 1938).

Noninfected egg yolk sac was prepared for inoculation of controls. The yolk sacs of 10 to 15-day incubated eggs were harvested and subjected to the same preparative procedures as the OCP inoculum.

All eggs used were verified to be free of bacterial con-

Table 1. Outline of experimental procedure



^aArticular tissues of 4 naturally infected and 2 intravenously inoculated lambs (1 control) were also examined.

taminants by incubation on blood agar at 37° C under aerobic and anaerobic conditions.¹

Cells

McCoy cells² were grown and maintained throughout the study at 37° C in Medium 199 containing 10% fetal bovine serum, 5% lactalbumin hydrolysate, and dihydrostreptomycin and kanamycin each at 100 µg/ml. Cell monolayers for inoculation were grown in roller tubes and in Leighton tubes containing glass coverslips. When the cells were nearly confluent, 0.2 ml of the OCP inoculum was added; after 1 hour of incubation, the excess inoculum was removed and new medium added. After incubation for 2, 8, 24, 48, 96, and 240 hours, the cells were removed from the culture tubes and frozen at -50° C or fixed as required for the various techniques. Cells were removed at additional postinoculation intervals for electron microscopy. Cells from at least 2 tubes at each post-inoculation time were examined by each procedure (Table 1).

¹Eggs were examined and found to be free of Mycoplasmata by Dr. K. R. Rhoades of the National Animal Disease Laboratory, Ames, Iowa.

²Monolayers of McCoy cells, ovine brain fibroblasts (Oa-1B), embryonic ovine testicle cells, and guinea pig peritoneal macrophages (Allison and Mallucci, 1965) were inoculated to determine which cell would best support growth of the OCP agent. McCoy cells were found, by immunofluorescent staining, to be relatively more susceptible and were used for the remainder of this study.

Controls for all procedures included noninoculated cells and cells inoculated with noninfected egg yolk sac. Control cells were removed from the tubes after 24 and 96 hours of incubation.

Animals

Two groups of lambs from a commercial source were used and maintained in isolation throughout the study. One group consisted of 12, 6 to 7-week-old Suffolk and Shropshire lambs (weanling lambs) and the other consisted of 8, 10 to 12-month-old Suffolk lambs (yearling lambs). All were serologically negative for antichlamydial antibodies by the complement fixation test (Meyer and Eddie, 1964).¹

Except for one control from each group, each lamb was exposed to the OCP agent by intraarticular inoculation. The right femorotibial and humeroradial joints of the weanling lambs and the right femorotibial joints of the yearling lambs were each injected with 0.5 ml of the OCP inoculum. The left femorotibial and humeroradial joints of 7 weanling lambs, including the control lamb, and the left femorotibial joints of 5 yearling lambs, including the control lamb, were each injected with 0.5 ml of noninfected egg yolk sac. An additional yearling lamb was inoculated with the OCP agent but did not become lame and was not included in this study.

¹Diagnostic antigen and known positive and negative serum controls were supplied by Dr. L. A. Page of the National Animal Disease Laboratory, Ames, Iowa.

Lambs were observed daily for signs of lameness and at predetermined intervals after inoculation they were killed with phenobarbital and necropsied immediately. Weanling lambs were killed 2, 4, 8, 14, 24, 48, and 72 hours and 6, 10, 12, and 24 days postinoculation. Yearling lambs were killed 8, 24, 48, 72, and 96 hours and 10 and 21 days postinoculation. The controls were killed after 24 hours (weanling lamb) and 10 days (yearling lamb).

Also included in this study were articular tissues from 4 naturally infected lambs, from 2 intravenously infected lambs, killed 11 days postinoculation, and from one noninoculated control lamb of the same origin as the lambs intravenously exposed.¹

Synovial and subsynovial tissues were taken from several of the most inflamed areas of each femorotibial and humero-radial joint of the weanling lambs and each femorotibial joint of the yearling lambs. Smears of synovial fluid from the same joints were made on glass slides. Specimens from the yearling lambs were divided and either frozen in a dry ice-alcohol mixture or fixed as required for the different histopathologic, histochemic, immunohistochemic, and electron microscopic techniques (Table 1). Specimens from the weanling lambs, naturally infected lambs, and intravenously infected lambs were divided and fixed in either formalin or glutar-

¹These tissues were supplied by Dr. L. A. Page.

aldehyde for histopathologic and electron microscopic examination.

Two additional weanling lambs were used for antichlamydial antibody production.

Histopathology and Histochemistry

The stains and procedures listed below were used to evaluate histopathologic alterations and to localize specific chemical substances and organisms in McCoy cell monolayers and in synovial tissues. Methods for determining enzymatic activities were followed as described by Barka and Anderson (1965).

1. Hematoxylin and eosin: Formalin-fixed cell monolayers, smears of synovial fluid, and paraffin sections of synovial membranes were stained with Harris' hematoxylin and counterstained with eosin Y (AFIP manual, 1960).
2. Giemsa: Formalin-fixed cells, smears of synovial fluid, and paraffin sections of synovial membranes were stained by the May-Grunwald-Giemsa technique (AFIP manual, 1960).
3. Alcian blue-periodic acid Schiff: Formalin-fixed specimens were treated with periodic acid, stained with Schiff's reagent and counterstained with Alcian blue (AFIP manual, 1960).

4. Oil red O: Staining was done on formalin-fixed cells and cryostat sections (Preece, 1965).
5. Acridine orange: Ribonuclease treated and nontreated cells and cryostat sections, fixed in an ethanol-acetic acid mixture, were stained with acridine orange (Pollard and Starr, 1962).
6. Acid phosphatase: Localization was by Gomori's lead method using β -glycerophosphate as the substrate at pH 5.
7. Alkaline phosphatase: Activity of this enzyme was determined by the Gomori-Takamatsu calcium method at pH 9.2 with β -glycerophosphate as the substrate.
8. Adenosine triphosphatase: Activity was determined according to the calcium method of Padykula and Herman with the substrate, adenosine phosphate, at pH 9.4.
9. Reduced diphosphopyridine nucleotide diaphorase: Nitro BT was used as the hydrogen acceptor according to the method of Scarpelli as modified by Barka and Anderson (1965).
10. Succinic dehydrogenase: Localization of activity was by the method of Nachlas, as modified by Barka and Anderson (1965), utilizing the tetrazolium salt, Nitro BT as the hydrogen acceptor and sodium succinate as the substrate.

11. Glucose-6-phosphate dehydrogenase: Activity was determined by the tetrazolium method of Nachlas as modified by Barka and Anderson (1965). Nitro BT was used as the hydrogen acceptor, with the substrate, glucose-6-phosphate.

Cell monolayers from each postinoculation interval and synovial tissues from each lamb were stained with hematoxylin and eosin, and Giemsa.¹ Synovial tissues of yearling lambs and McCoy cells were also stained with Alcian blue-periodic acid Schiff, oil red O, and acridine orange (Table 1).

All enzymatic activities were determined on unfixed frozen specimens of synovial tissues from yearling lambs and McCoy cells. Infected and noninfected specimens were stained in the same solutions. Substrate-deficient control solutions were used for each enzyme; selected specimens were incubated in solutions in which the respective substrate had been omitted.

¹Preliminary staining of infected cell cultures and egg yolk sacs by Castaneda's method (Meyer and Eddie, 1964), by Pinkerton's modification of Macchiavello's method (AFIP manual, 1960), and by Giménez's method (1964) proved inferior to Giemsa for detection of organisms and evaluation of cellular changes. Organisms were easiest to find in eggs where they occurred as compact intracytoplasmic inclusions. Similar trials indicated phase microscopy was not satisfactory for detection of organisms in cell cultures because intracytoplasmic granules were present in cells inoculated with either the OCP agent or egg yolk sac. Such granules probably were phagocytized egg yolk material. Similar results were reported by Officer and Brown (1960) with the agent of psittacosis.

Ultraviolet light for examination of acridine orange stained slides was from a Leitz mercury vapor lamp equipped with a blue exciter filter (BG-12) and a blue absorption filter.

Immunohistochemistry

McCoy cells representing each postinoculation interval and synovial tissues from yearling lambs were stained by fluorescent antibody and enzyme antibody techniques. Synovial tissue from only one lamb was stained by the enzyme antibody technique.

Sheep antichlamydial antibody was prepared by exposing 2 lambs to the virulent organism, followed in 2 weeks by 3 biweekly intramuscular injections of a mixture of equal parts of the OCP inoculum and Freund's complete adjuvant (Difco, Inc., Detroit, Michigan).¹ The lambs were killed 2 weeks after the last injection and the antibody titer determined by serum neutralization (Cunningham, 1966). The serum with the highest titer (Neutralization Index = 4.4) was used for immunohistochemic procedures.

Fluorescent antibody techniques were, with minor modifi-

¹In an effort to obtain heterogeneous systems for use with immunohistochemical procedures, an unsuccessful attempt was made to establish persistent infection of the OCP agent in McCoy cells. After 4 serial passages, cell monolayers were noninfected as determined by fluorescent antibody staining.

cations, according to the methods of Coons (1958). The hyper-immunized lamb was killed, the serum harvested, and the gamma-globulins precipitated with equal parts of saturated ammonium sulfate. Ammonium sulfate was removed from the precipitate by dialyzing with 0.85% sodium chloride. The gamma-globulins were conjugated with fluorescein isothiocyanate, dialyzed, and stored at -50° C in 2 ml aliquots.

For enzyme antibody staining, the peroxidase method of Nakane and Pierce (1967) was employed. Horseradish peroxidase (Nutritional Biochemical Corporation, Cleveland, Ohio) was conjugated with ammonium sulfate precipitated gamma-globulins using p,p'-difluoro-m-m'-dinitrodiphenyl sulfone (General Biochemicals, Chagrin Falls, Ohio). The conjugate was divided into one ml amounts and stored at -50° C until used.

Before use, both conjugates were absorbed with dried, acetone-extracted rabbit-liver and egg-yolk-sac powders prepared according to the method of Coons (1958). Cells on coverslips and cryostat synovial sections were fixed in acetone,¹ air dried, flooded with rabbit anti-egg-yolk-sac serum and incubated at 37° C for 30 minutes before staining. The anti-egg-yolk-sac serum was prepared in rabbits by 3 biweekly intramuscular injections of noninfected egg yolk sac in Freund's complete adjuvant and the serum harvested 2 weeks

¹Antigenic specificity of the OCP agent as determined by fluorescent antibody staining was not retained after fixation in 95% ethanol, 10% formalin, or 2.5% glutaraldehyde.

after the last injection. After removal of the rabbit serum by washing in 0.85% sodium chloride, the specimens were flooded with the respective antichlamydial conjugates and incubated at 37° C for 30 minutes.

Controls, in addition to noninfected specimens, consisted of selected specimens incubated with nonconjugated antichlamydial globulins prior to application of each conjugate.

Specimens stained with the fluorescein conjugate were observed immediately after staining using a Leitz mercury vapor light source with a UV-UG1 exciter filter and a UV absorption filter.

Sections of synovium to be stained with the conjugates were selected by staining adjacent sections with hematoxylin and eosin. Sections containing excess fat or fibrinous exudate were discarded. Subsequent to fluorescent antibody staining, coverslips were removed from all specimens and they were stained with Giemsa for further evaluation of fluorescent particles.

Electron Microscopy

Infected cells were examined after 1, 2, 4, 8, 16, 24, 48, 72 and 96 hours of incubation and noninfected cells after 24 and 96 hours of incubation. Cells were removed from roller tubes with 0.25% trypsin in 0.85% saline, washed in sodium cacodylate buffer (0.2M, pH 7.4), fixed in 2.5% glutaraldehyde, embedded in agar, postfixed in 1% osmium tetroxide,

dehydrated in a graded series of ethanol, and embedded in epoxy resin (Epon 812: Shell Chemical Company, San Francisco, California) according to the method of Luft (1961).¹

Synovial tissues and fluid from all lambs, except 2 naturally infected lambs, were examined by electron microscopy. Synovial fluid was fixed in glutaraldehyde and processed in the same manner as cell cultures. The synovial membranes were cut in 1 to 2 mm squares and processed likewise, except the agar embedment was omitted. For orientation with the light microscope, one micron sections of synovial membranes were cut and stained with paragon (Paragon C. and C. Company, Inc., New York, New York) according to the method of Martin et al. (1966).

Ultrathin sections were stained with uranyl acetate and lead citrate (Venable and Coggeshall, 1965), then examined with a Philips model 200 electron microscope.

¹Selected cell cultures infected with the OCP agent were also processed by the enzyme antibody method of Nakane and Pierce (1967) for electron microscopy. Acetone was used as the fixative. This procedure was not satisfactory; particles of the agent in enzyme antibody stained and unstained sections were indistinguishable.

RESULTS

McCoy Cell Cultures

Cytopathology

Hematoxylin and eosin Monolayers of infected cells, stained with hematoxylin and eosin, appeared unaffected after 48 hours of incubation. At 96 hours postinoculation, multiple foci from which cells had detached from the glass were present and at 240 hours, most cells were floating free in the medium.

Giemsa Giemsa staining revealed a few cells in infected monolayers with blue intracytoplasmic granules of the OCP agent (Figure 1). None were observed prior to 48 hours postinoculation, at which time only a few organisms were found. After 96 hours the organisms were more numerous and were usually diffusely distributed in the cytoplasm. Less frequently, dense aggregates of organisms were found. Similar inclusions were not found in control monolayers.

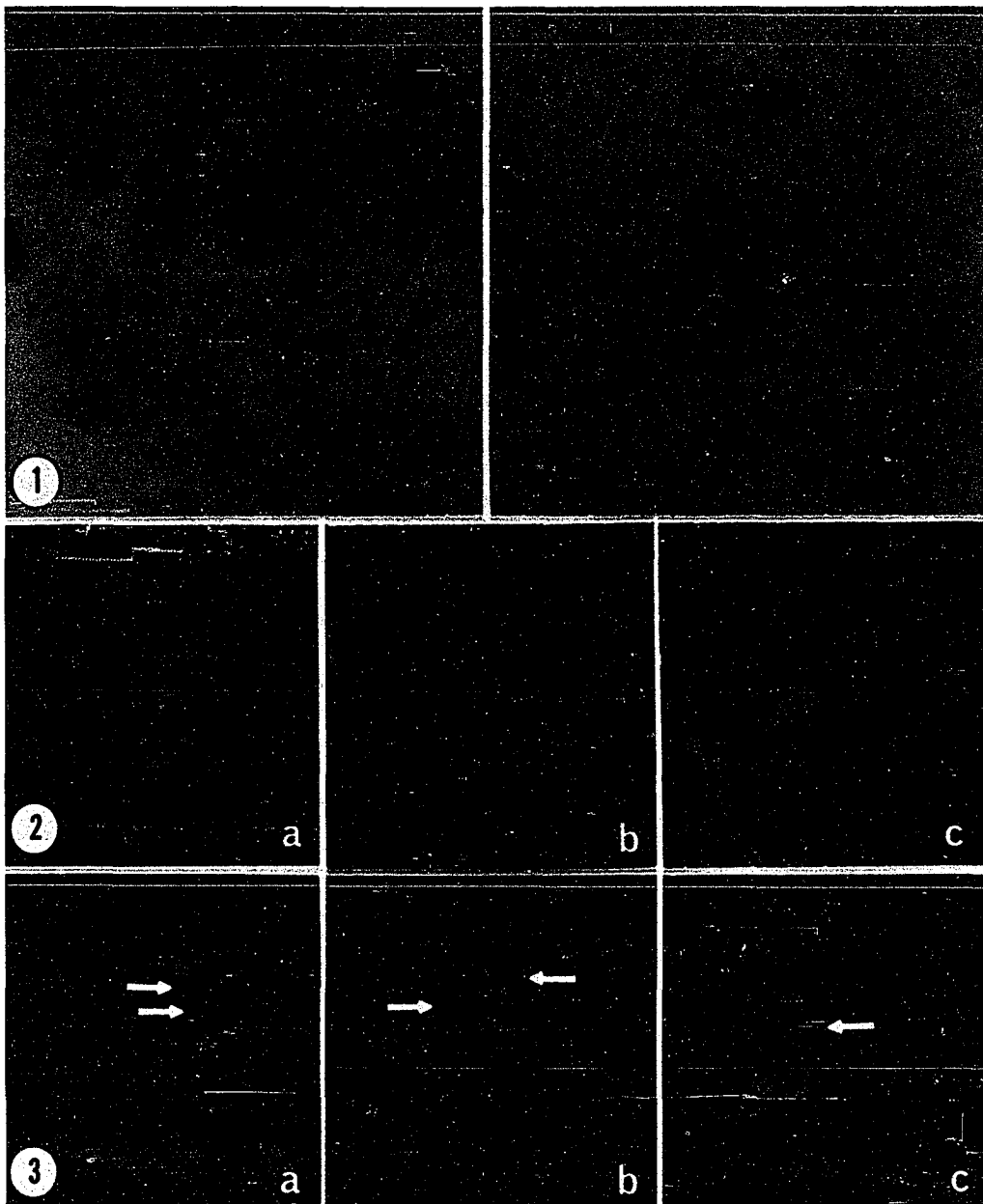
Cytochemistry

Acridine orange With acridine orange staining of infected cell monolayers, many intracytoplasmic foci of reddish-orange fluorescence were present after 24 hours of incubation (Figure 2). These fluorescent foci were smallest in 24-hour cultures and gradually increased in size with an increase in incubation time. After 96 hours, the entire cytoplasm of many cells fluoresced a bright reddish-orange while

Figure 1. Giemsa-stained-McCoy-cell monolayers 96 hours after inoculation with the OCP agent. (a) Organisms diffusely scattered in the cytoplasm of a cell and (b) in a compact intracytoplasmic inclusion in a mitotic cell. X 500

Figure 2. McCoy-cell monolayers stained with acridine orange, (a) 24 hours, (b) 48 hours, and (c) 96 hours after inoculation with the OCP agent. Orange cytoplasmic inclusions were smallest at 24 hours, larger at 48 hours, and filled the cytoplasm at 96 hours postinoculation. Note the similarity of color, indicating ribonucleic acid, regardless of the postinoculation interval. X 500

Figure 3. McCoy-cell monolayers stained with acridine orange subsequent to treatment with ribonuclease, (a) 24 hours, (b) 48 hours, and (c) 96 hours after inoculation with the OCP agent. Green inclusions (arrows), indicating deoxyribonucleic acid, were similar in size and location to orange inclusions without ribonuclease treatment in Figure 2. X 500



fluorescent foci similar in size and color to those in 24-hour cultures were present in other cells. These foci appeared as granular masses without definite borders, particularly in older cultures, but were similar in color regardless of age. Acridine orange staining of infected monolayers subsequent to treatment with ribonuclease caused green fluorescence of cytoplasmic inclusions identical in size and distribution to those without ribonuclease treatment (Figure 3). However, green fluorescent inclusions were less distinct than orange ones. Control monolayers did not fluoresce with the characteristic inclusions of infected cells.

Alcian blue-periodic acid Schiff and oil red O Differences were not found between infected cells and cells to which noninfected egg yolk sac had been added when stained with oil red O or Alcian blue-periodic acid Schiff. Oil red O staining material was found adhering to the surface of cells and in small amounts within the cytoplasm. Noninoculated cells did not stain with oil red O. The cytoplasm of all cells, infected and controls, stained faintly with periodic acid Schiff and contained multiple granules which stained with Alcian blue. Staining was unaffected by treatment with diastase.

Acid phosphatase Inoculated and noninoculated cells were similar in acid phosphatase activity. Cytoplasmic staining was diffuse, frequently with heavier concentrations at one pole of the cell. Substrate-deficient controls were

negative.

Alkaline phosphatase Following treatment to detect alkaline phosphatase activity, a fine precipitate was present on all infected and noninfected cells, and on the substrate-deficient controls. Specific alkaline phosphatase activity was not detected.

Adenosine triphosphatase Cobalt sulfide precipitate, indicating adenosine triphosphatase activity, was diffuse throughout the cells. Intense nuclear staining was attributed to diffusion artifact. Difference was not detected in infected and noninfected cells. Substrate-deficient controls were negative.

DPNH diaphorase Activity of this enzyme was confined to the cytoplasm of cells and was equal in infected and noninfected cells. Substrate-deficient controls were negative.

Succinic dehydrogenase Formazan was deposited irregularly throughout the infected cell monolayers; however, because of similarity to controls, this could not be attributed to infection. Staining was light and localized in the cytoplasm.

Glucose-6-phosphate dehydrogenase Cytoplasmic staining was diffuse in all cell monolayers to which the OCP inoculum or noninfected egg yolk sac had been added and occurred with less intensity in the cytoplasm of noninoculated cells. The difference was apparently related to the presence of egg yolk material.

Immunohistochemistry

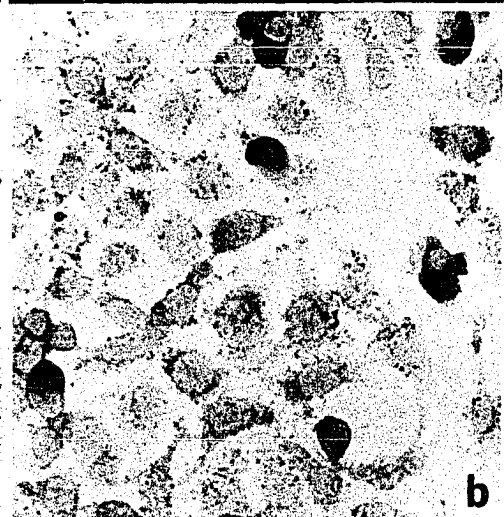
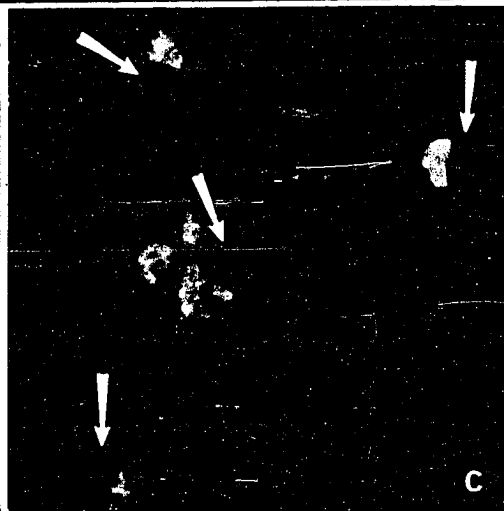
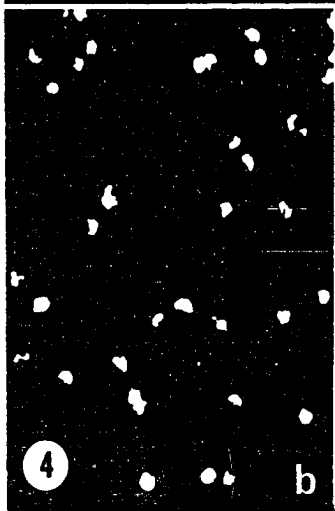
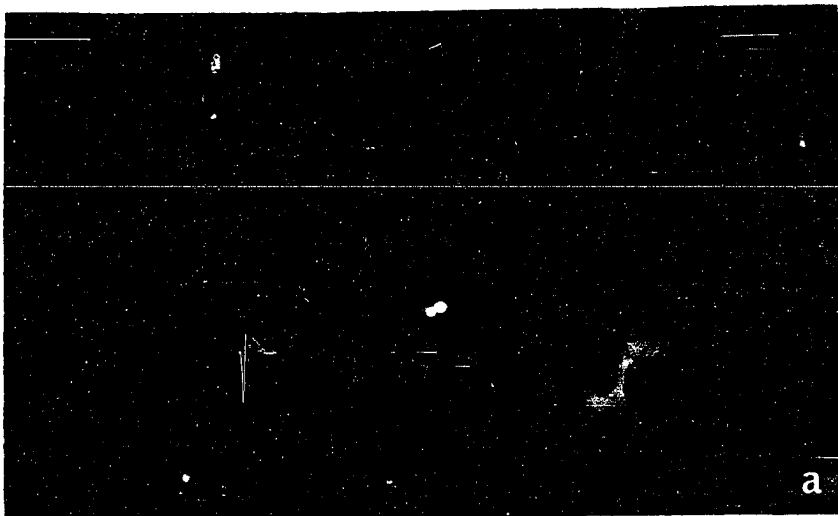
Green fluorescent inclusions were present in the cytoplasm of OCP-infected cells incubated for 24, 48, 96, and 240 hours and stained by the fluorescent antibody technique (Figure 4). An estimated 10% of the cells contained fluorescent inclusions 24 and 48 hours after inoculation. At 96 hours, approximately 15% of the cells fluoresced and at 240 hours, most of the cells still adhering to the glass contained fluorescent inclusions. Minute fluorescent granules in cells prior to 24 hours of incubation were indistinguishable from controls. The inclusions, smallest at 24 hours postinoculation, increased in size to occupy most of the cell cytoplasm 96 and 140 hours after inoculation. They were composed of many small fluorescent particles. In addition to the large inclusions after 96 hours, many cells contained small inclusions similar to those at 24 hours postinoculation.

Staining of chlamydial inclusions with peroxidase-antibody conjugate was satisfactory only after 96 hours of incubation (Figure 5). The dark brown reaction product was identical in distribution to the large inclusions with fluorescent antibody staining. Smaller chlamydial inclusions, if present, were hidden by nonspecific stain precipitate.

Specificity of immunohistochemical staining was verified by blocking the reactions with nonconjugated-ovine-antichlamydial globulin and nonreactivity of noninfected cell monolayers. Substrate-deficient controls for the peroxidase

Figure 4. Chlamydial-infected McCoy cells (a) 24 hours and (b and c) 96 hours postinoculation. Stained with fluorescein-isothiocyanate-conjugated-antichlamydial antibody. Note the discrete cytoplasmic fluorescence at 48 hours and diffuse cytoplasmic fluorescence at 96 hours. Arrows indicate the nuclei of the fluorescent cells. a. and c. X 500; b. X 125

Figure 5. Ninety-six-hour chlamydial-infected McCoy cells after staining with horseradish peroxidase-antibody conjugate. Dark brown to black cytoplasmic staining indicates the presence of organisms. a. X 50; b. X 320



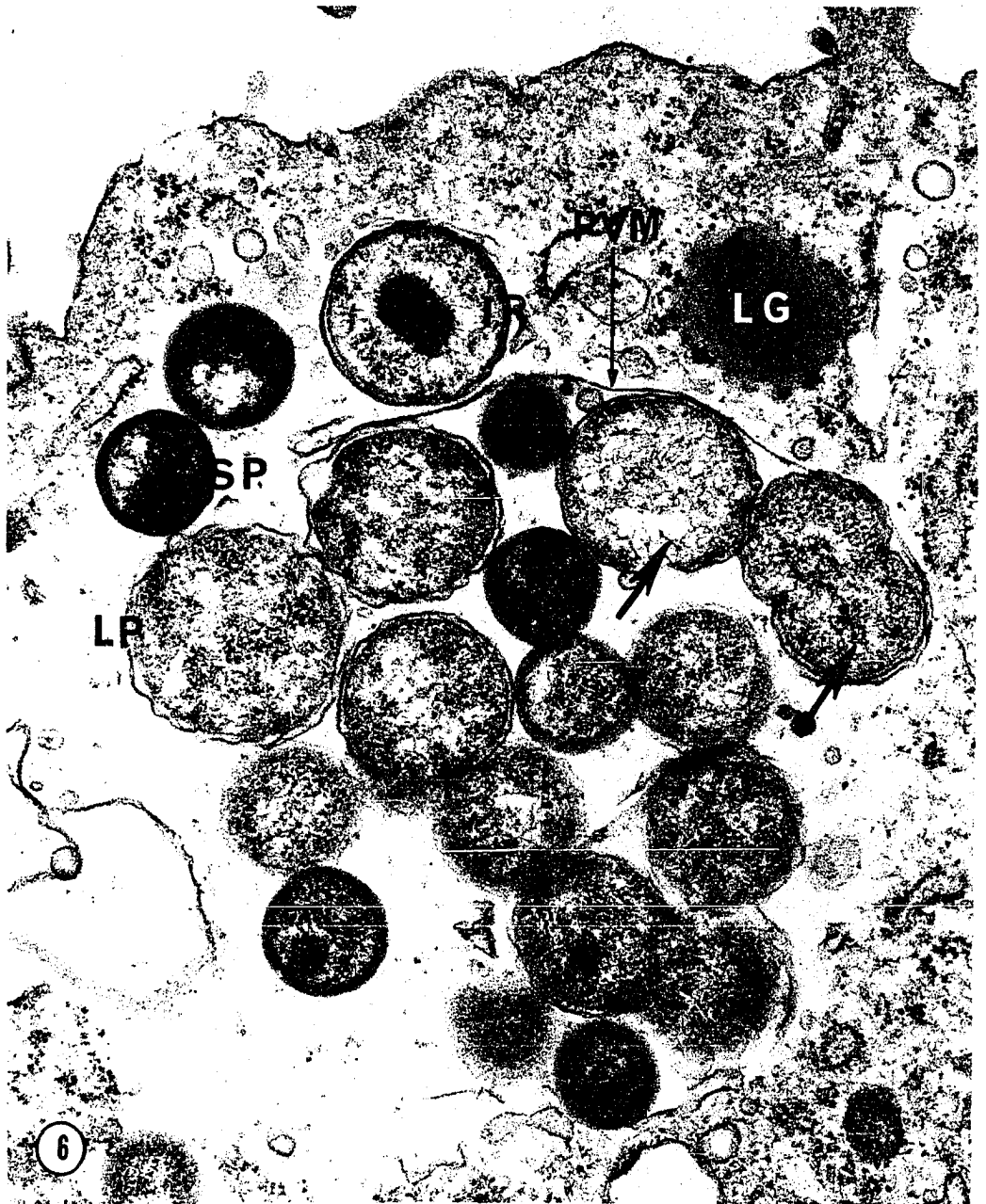
reaction were also negative.

Electron microscopy

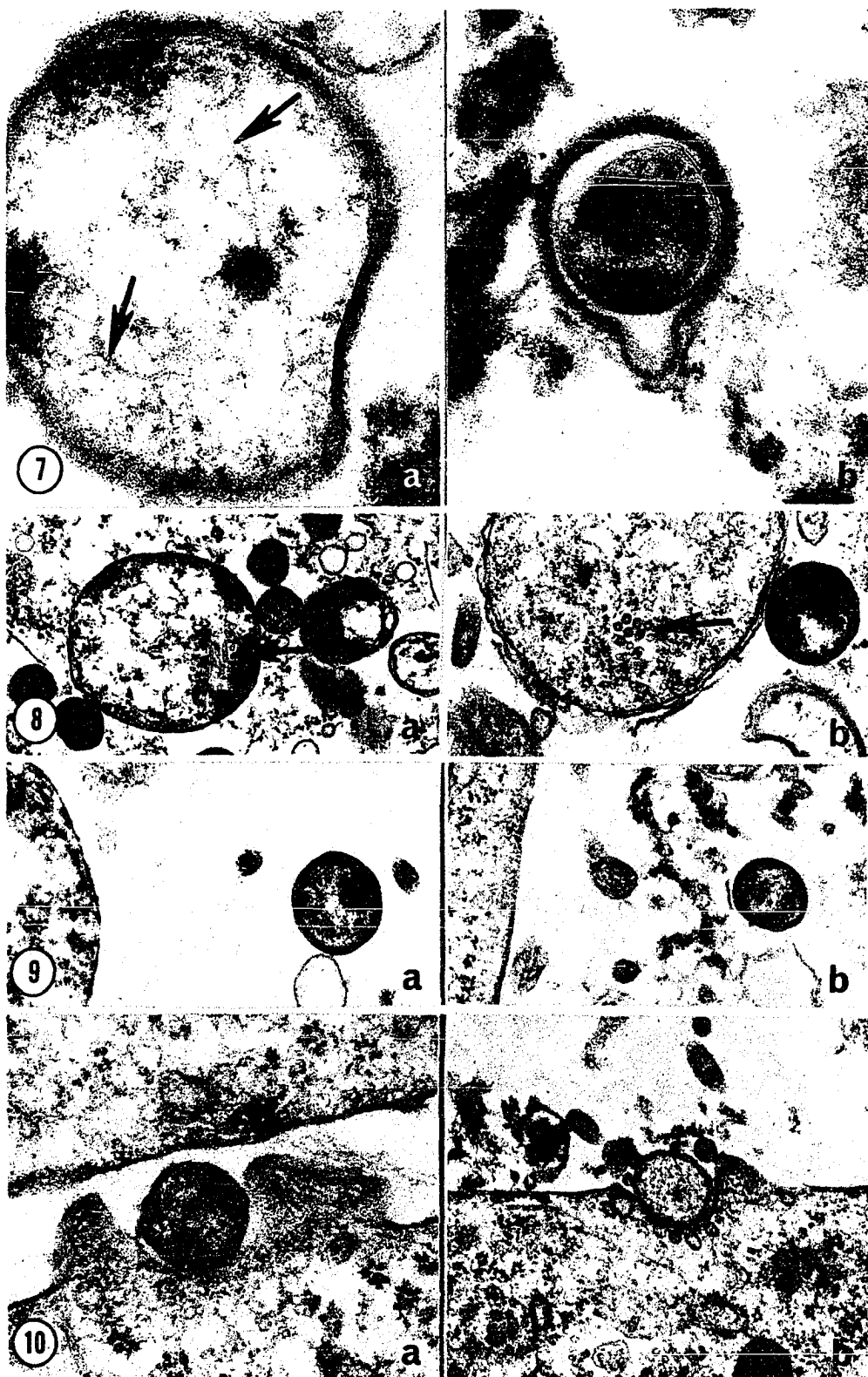
Chlamydial organisms were found either in the extracellular fluid or in the cell cytoplasm of all cultures inoculated with the OCP agent. Structure of the agent varied from small dense particles (small particles) with apparent rigid cell walls to large granular particles (large particles) of less rigidity and electron density. Small particles were spherical, from 250-450 nm in diameter, with an eccentrically placed electron-dense nucleoid and tightly invested by a multilaminated wall (Figure 6). The wall appeared to be composed of 4 electron-dense laminae separated by electron-lucent laminae; i.e., 2 unit membranes. This was more distinct in organisms found in the extracellular fluid (Figure 7). Particles (intermediate particles), intermediate in size and structure to the small and large particles, were from 400-600 nm in diameter, had a centrally-placed nucleoid and were enveloped by either a rigid cell wall or by 2 unit membranes (Figures 6 and 13). Large particles varied in size from 600-1500 nm; however, most were from 800-1200 nm in diameter (Figure 6). Internal structure consisted primarily of small granules; many also contained electron-dense strands of deoxyribonucleic acid. A few had internal structures which appeared either as microtubules (Figure 8) or as unit membranes which divided the particles into 2 or 3 portions (Figure 13).

Figure 6. Electron micrograph of a chlamydial inclusion in the cytoplasm of a McCoy cell 48 hours after inoculation. The various stages of the developmental cycle are recognizable; small particle (SP), intermediate particle (IP), and large particle (LP). Respective sizes are 410, 630, and 740 nm. Note the dumbbell shape of some large particles, indicative of binary fission. The multilaminated nature of the wall of the small particles, double unit membrane surrounding the intermediate and large particles, and fragments of the plasma membrane of the phagocytic vacuole (PVM) are visible. Strands of deoxyribonucleic acid (arrows) are present in large and intermediate particles. Uranyl acetate and lead citrate. X 41,000

IP - intermediate chlamydial particle
LG - lipid globule
LP - large chlamydial particle
PVM - phagocytic vacuole membrane
SP - small chlamydial particle
Arrows - strands of deoxyribonucleic acid



- Figure 7. Electron micrographs of chlamydial organisms found in the extracellular fluid of McCoy cell cultures. The walls appear to be composed of 2 distinct unit membranes. Fibrous structures (arrows) are interpreted as deoxyribonucleic acid. Uranyl acetate and lead citrate.
a. and b. X 92,000
- Figure 8. Electron micrographs of intracytoplasmic chlamydial particles containing microtubules (arrows). Uranyl acetate and lead citrate.
a. X 24,000; b. X 41,000
- Figure 9. Electron micrographs of (a) one and (b) two-hour McCoy cell cultures containing extracellular chlamydial organisms (a) with and (b) without a visible nucleoid. The multilaminated structure of the wall is apparent. Uranyl acetate and lead citrate.
a. X 41,000; b. X 34,000
- Figure 10. Chlamydial organisms partially surrounded by cytoplasmic projections of McCoy cells after 8 hours of incubation. Uranyl acetate and lead citrate.
a. X 72,000; b. X 34,000



Large particles were surrounded by 2 unit membranes. All chlamydial forms contained small (16-24 nm) unattached, electron-opaque structures characteristic of ribosomes.

Prior to 8 hours of incubation, chlamydial organisms were found only in extracellular fluid, either as discrete small particles or as slightly larger particles without detailed internal structure (Figure 9). These organisms were structurally identical to organisms in the inoculum and were partially enveloped by cell membrane in 8-hour cultures (Figure 10). Intracellular organisms were found after 16 hours of incubation in phagocytic vacuoles or partially surrounded by membranous projections (Figure 11). At 24 hours, small particles were seen in the process of being engulfed by cells and large particles, either singular or in clusters of 2-3, were scattered throughout the cytoplasm (Figure 12). Intact or broken remnants of plasma membrane of the phagocytic vacuoles surrounded the inclusions. After 48 hours of incubation, aggregates of organisms, predominantly large particles, were found in many cells (Figure 13). Many of the large particles were in various stages of division. Small and intermediate particles were present in fewer numbers. The particles were free in the cytoplasm with only remnants of the phagocytic-vacuole membrane remaining. Seventy-two and 96 hours postinoculation, small particles predominated, but many large and intermediate particles were present (Figure 14). Rents in the plasma membrane with loss

Figure 11. Electron micrographs of McCoy cells containing chlamydial particles after 16 hours of incubation. Small forms are present (a) in a plasma membrane invagination, and (b) in a cytoplasmic vacuole, and (c) a larger particle is present in the cytoplasm. Uranyl acetate and lead citrate.
a. and b. X 41,000; c. X 18,000

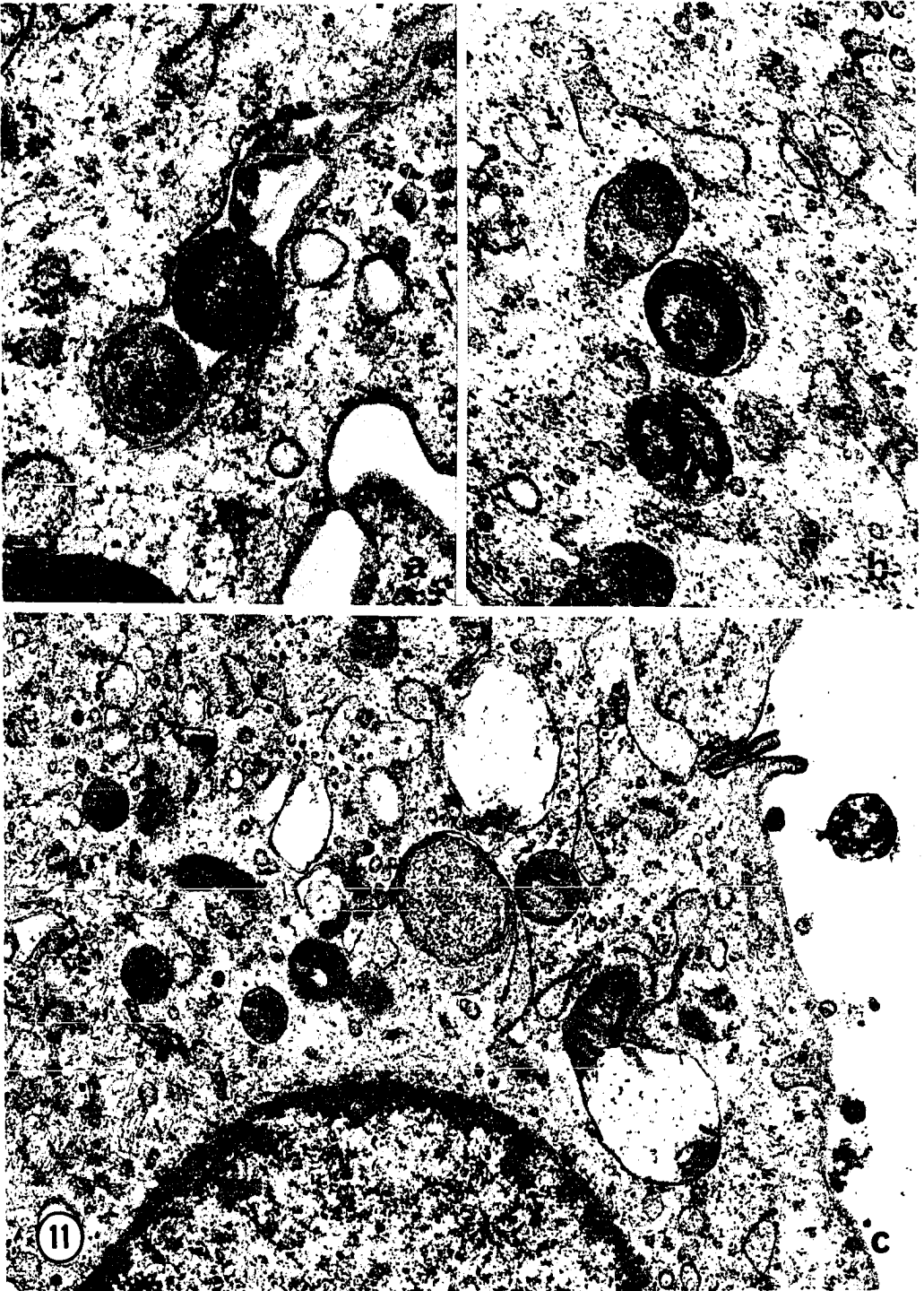


Figure 12. Electron micrographs of McCoy cells 24 hours after inoculation with the OCP agent, (a) a small particle appears in the process of being phagocytized by a cell, (b) a large particle surrounded by the intact membrane of the phagocytic vacuole in the cytoplasm of a cell, and (c) 3 intracytoplasmic organisms surrounded by broken fragments of the phagocytic-vacuole membrane (arrows). Uranyl acetate and lead citrate.
a. X 34,000; b. and c. X 24,000

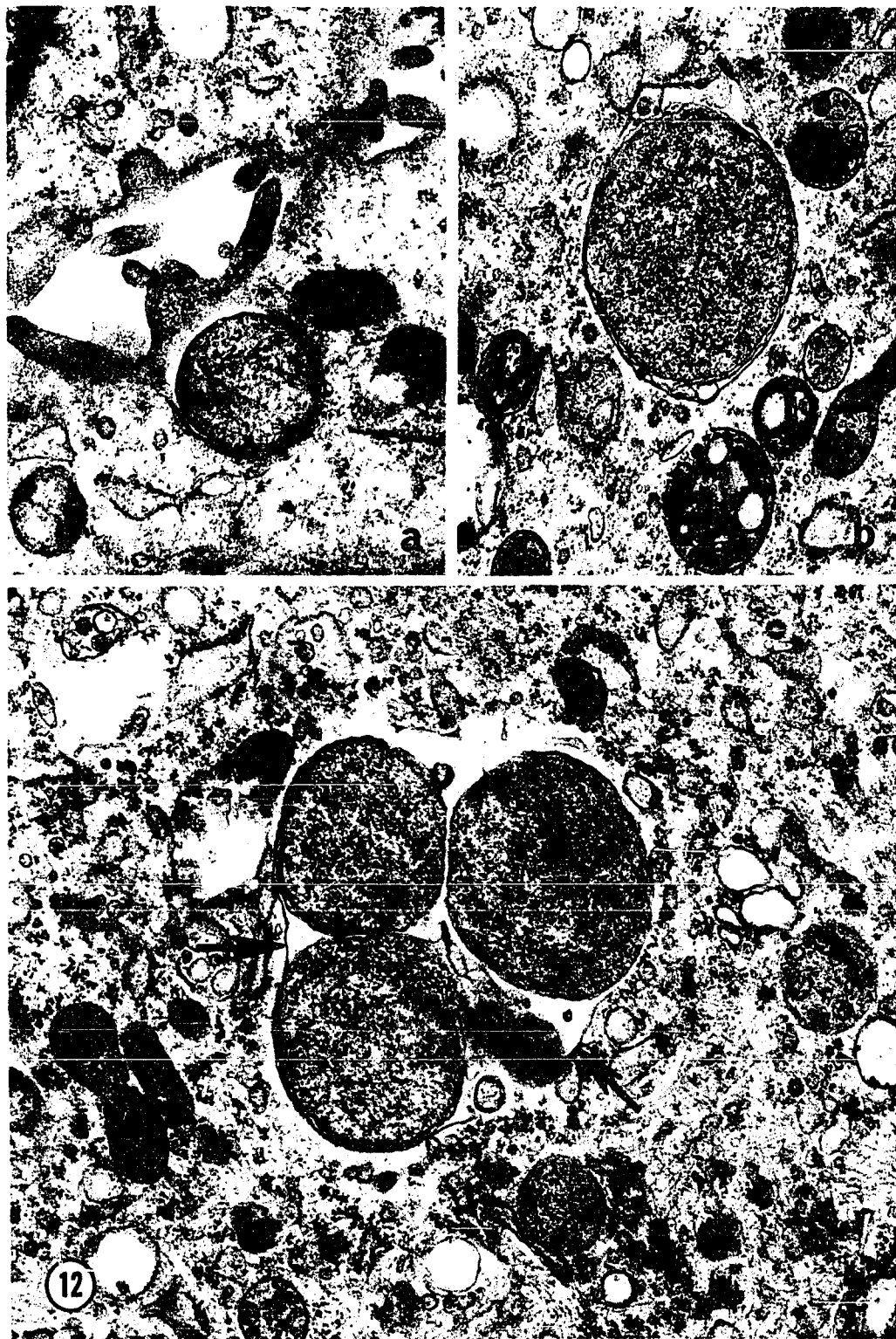


Figure 13. Cytoplasm of a McCoy cell containing various stages, predominantly large particles, of the OCP agent. Unit membranes divide several of the large particles into 2 or more portions. After 48 hours of incubation. Uranyl acetate and lead citrate. X 11,000

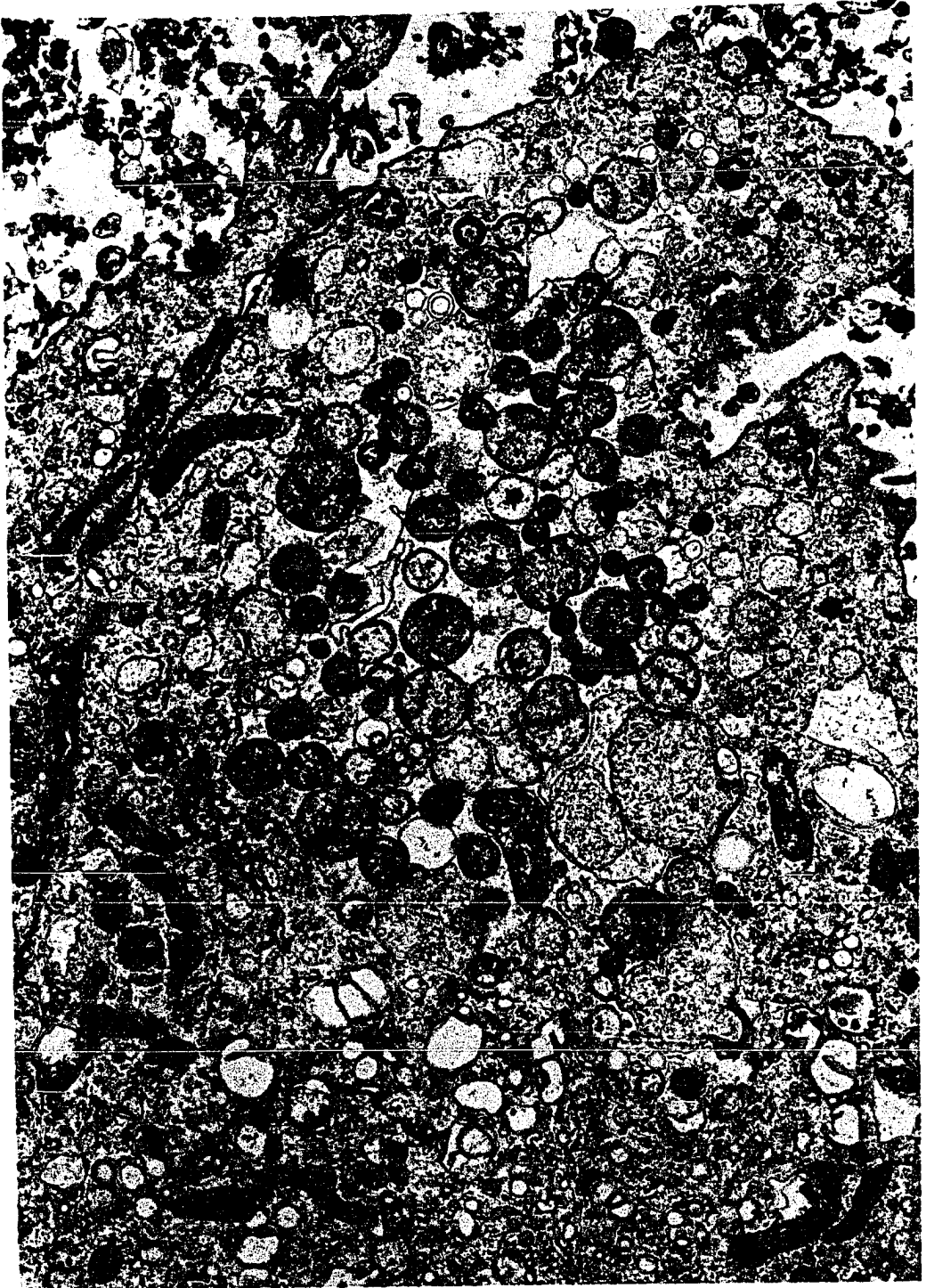
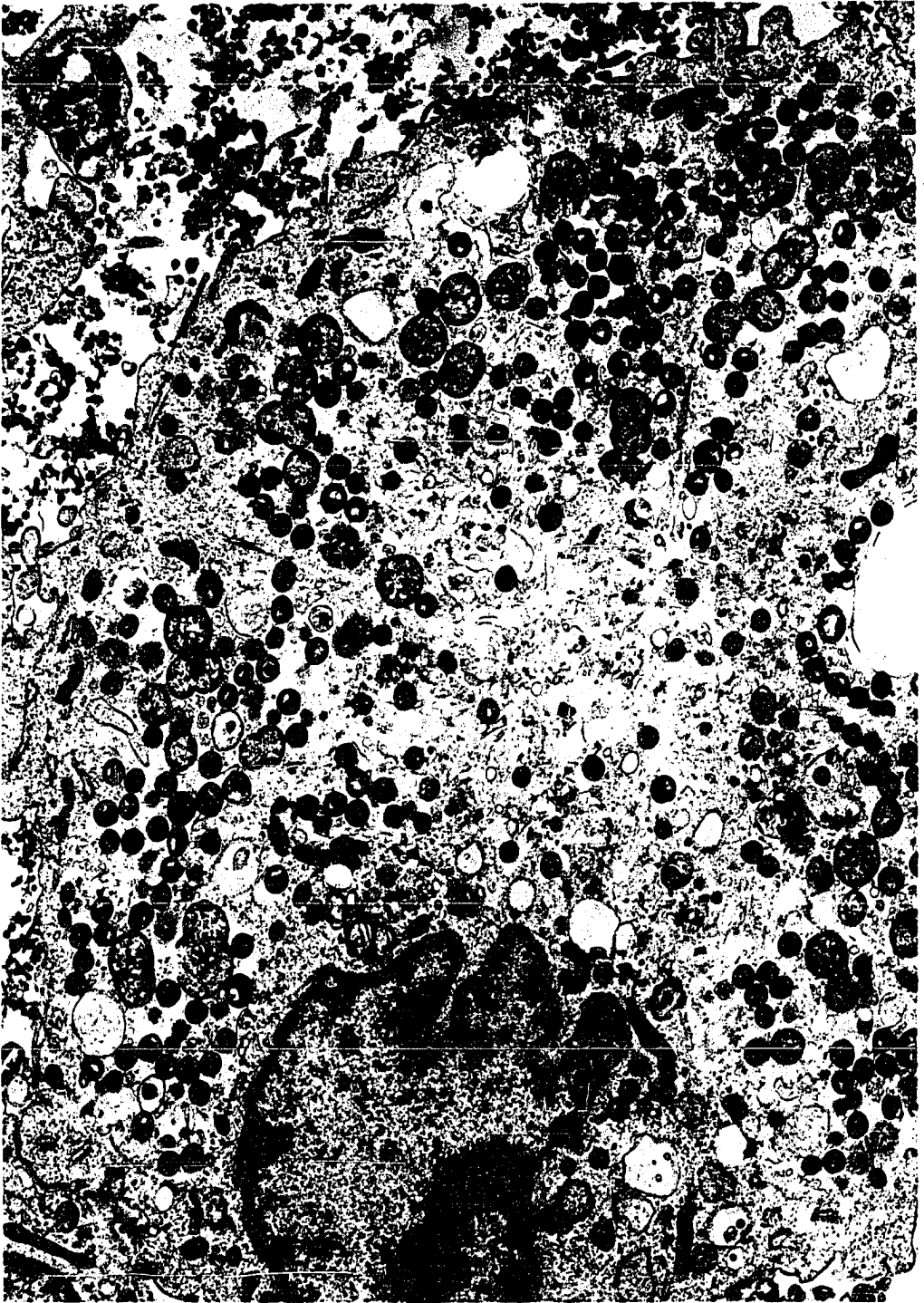


Figure 14. Electron micrograph of a chlamydial-infected McCoy cell 72 hours after inoculation. Numerous organisms of various size, predominantly small particles, are scattered throughout the cytoplasm. Several large particles are dumbbell shape, indicative of binary fission. Uranyl acetate and lead citrate. X 8,700



of cytoplasmic constituents and chlamydial organisms were evident in many cells (Figure 15). In a few cells incubated for 96 hours, small inclusions of large particles, similar to those in 24-hour cultures, were found.

In addition to chlamydiae, numerous viral particles typical of type C murine leukemia virions, as classified by Bernhard (1960), were found within and surrounding the cells in all McCoy cell cultures examined (Figure 16). The majority were located in the extracellular space. Intracytoplasmic virions were within membrane-lined vacuoles which may represent invaginations of the plasma membrane. An occasional cytoplasmic projection (virus bud) was present which contained a partially formed virus particle. These buds were found in both the membranes of cytoplasmic vacuoles and the external limiting membranes of cells.

The ultrastructure of the viral particles, except the budding form, was similar regardless of location. They consisted of a moderately electron-dense central nucleoid core surrounded by an electron-lucent zone and an envelope. The particles were from 90 to 100 nm in diameter.

Lamb Articulations

Clinical response

All lambs included in this study, which received the OCP inoculum intraarticularly, became severely lame within 24 hours and remained so until killed. No other signs of

Figure 15. A McCoy cell 96 hours after inoculation with the agent of OCP. Ruptured cell membrane has released the numerous intracytoplasmic organisms. Uranyl acetate and lead citrate.
X 8,700

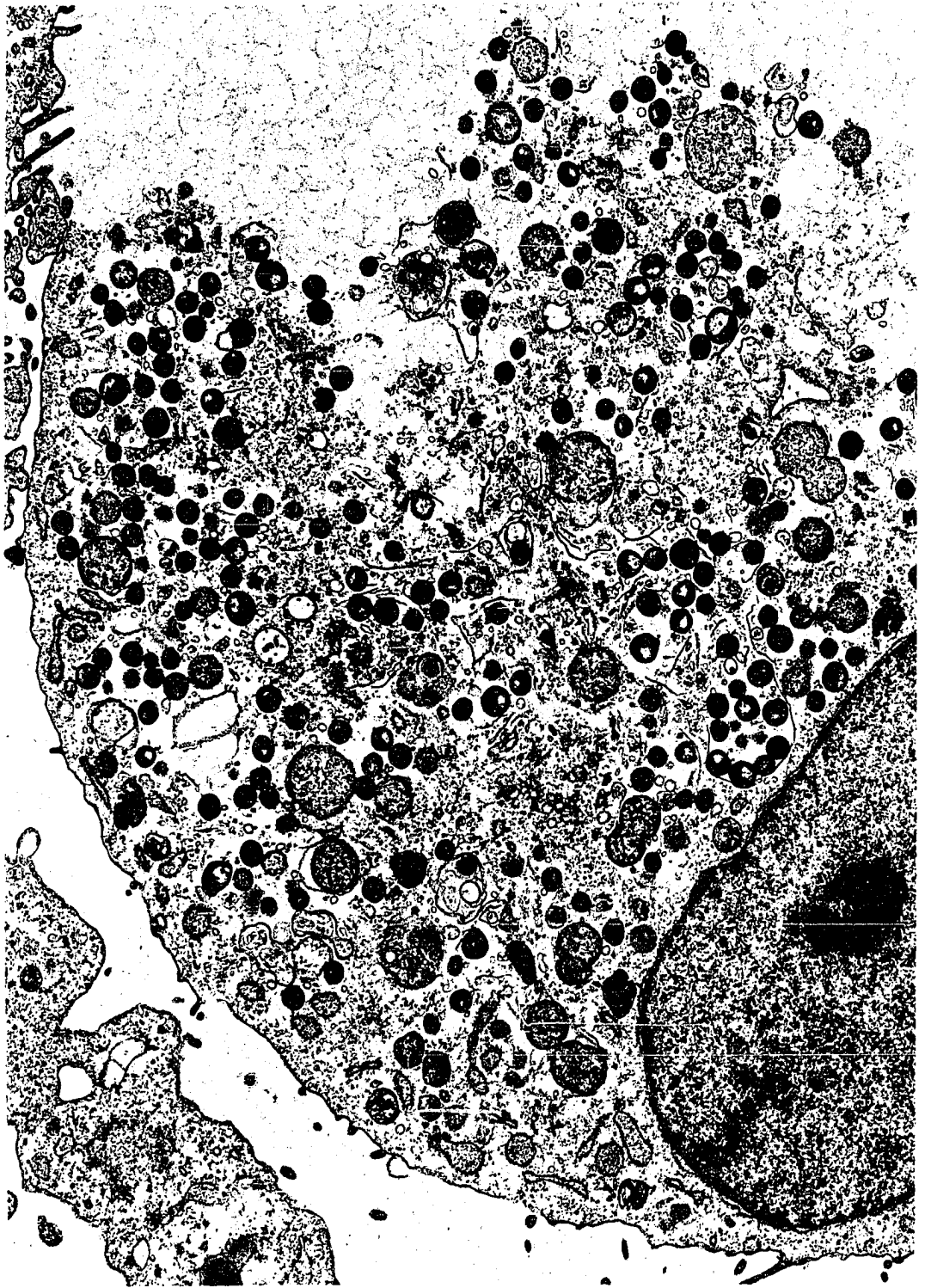
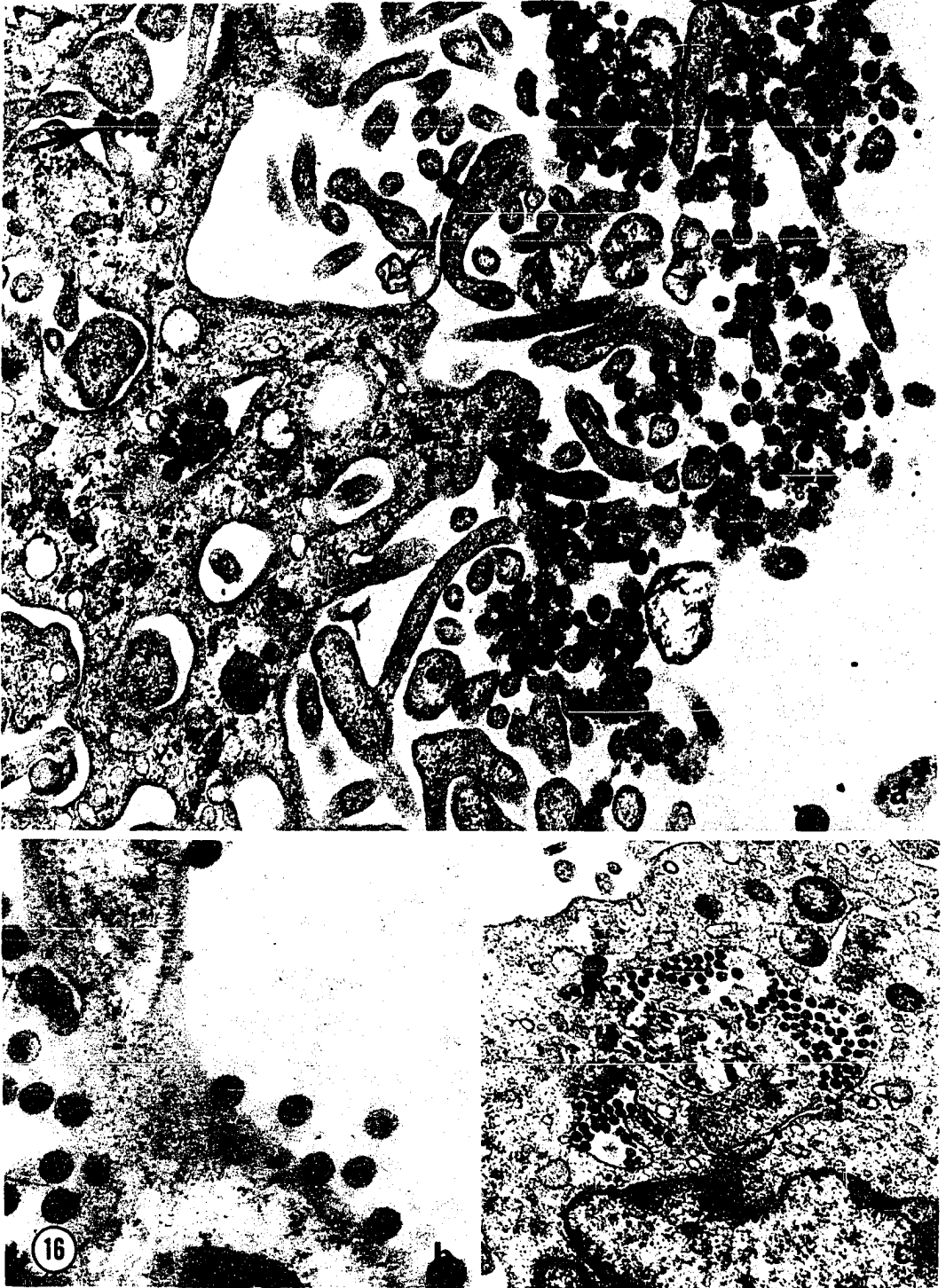


Figure 16. Electron micrographs of 8-day cultures of McCoy cells containing type C particles resembling murine leukemia virus. Virions are seen (a) in aggregates at the cell surface and incompletely formed in the plasma membrane as a viral bud (arrow), (b) attached to the cell surface, and (c) within vacuoles which probably represent invaginations of the plasma membrane. Uranyl acetate and lead citrate.
a. X 34,000; b. X 41,000; c. X 18,000



disease were seen. Lameness was not observed in noninoculated joints or in joints into which egg yolk sac had been injected.

Macroscopic lesions

A consistent difference was not apparent between articular lesions of weanling and yearling lambs; therefore, the 2 groups will be considered together. Neither was there a difference between the inflammatory reactions in the femorotibial and humeroradial joints of weanling lambs.

In joints of lambs killed 2-14 hours after injection of the OCP agent, there were hyperemic synovial membranes and excessive joint fluid. From 2-6 ml of a straw-colored fluid, containing an occasional strand of fibrin, were aspirated from each inflamed joint.

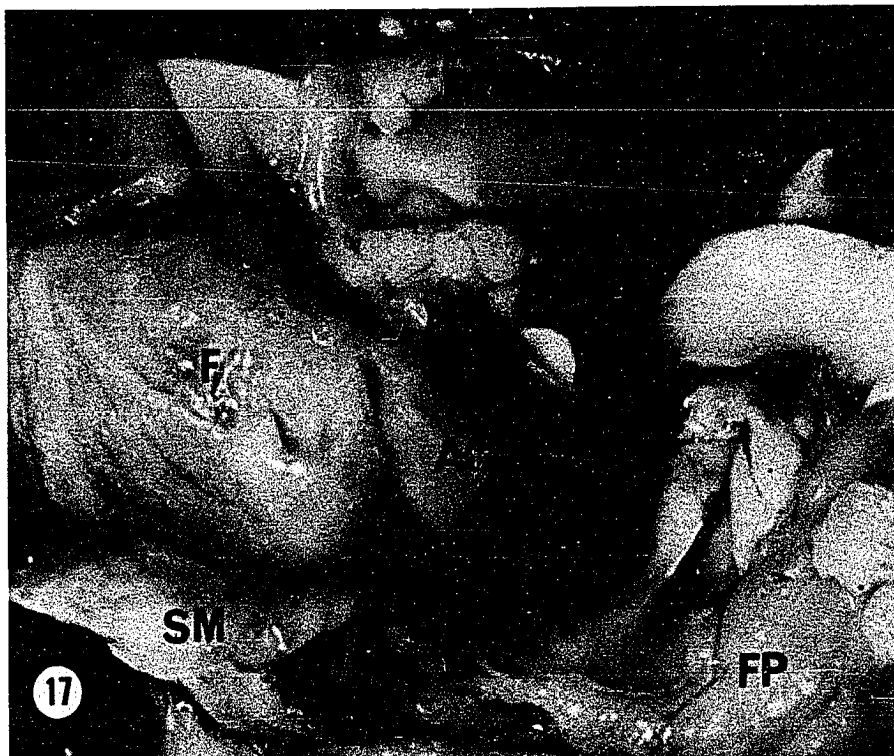
Infected joints of lambs killed after 24 hours contained excess fluid and large sheets of fibrin which covered most of the synovial surfaces. It was especially prevalent around tendons and under patellae. A few petechia were present in the synovial membranes.

The joints of lambs infected for 48, 72, 96, and 144 hours were similar to those infected for 24 hours; however, there was more fibrin (Figure 17) and the synovial fluid was grayish-yellow and of greater viscosity, thus resembling a mucopurulent exudate. Joints infected for 10 and 12 days were likewise affected, except the fibrin was firmly adhered to the underlying tissues. The femorotibial joint of one lamb, killed 12 days after inoculation, was slightly enlarged

Figure 17. Femorotibial joint of a lamb killed 72 hours after being inoculated with the OCP agent. Large sheets of loose fibrin (F) cover much of the joint surface

Figure 18. Femorotibial joint of a lamb killed 21 days after being inoculated with the OCP agent. Partially organized fibrin (OF) is firmly adhered to the underlying tissue

AC - articular cartilage
F - fibrin
OF - organized fibrin
SM - synovial membrane
FP - fat pad



as a result of the articular exudate and periarticular edema.

Synovial membranes of lambs, killed 21 and 24 days after inoculation, were thickened by fibrous connective tissue and covered by partially organized fibrin (Figure 18). They were hyperemic and small hyperplastic villi projected into the joint spaces, frequently as flattened fronds extending over the articular cartilages. The synovial fluid was very viscous but only slightly excessive.

The noninoculated joints of the 2 control lambs, the noninoculated joints of lambs given the OCP agent and the joints examined 24 hours or more after egg yolk sac was injected were normal in gross appearance. Joints of lambs that had received egg yolk sac and examined no later than 24 hours postinoculation had excess normal appearing synovial fluid. Except in the joints examined at 2 hours postinoculation, the quantity of fluid was less than that in the contralateral joints infected with OCP.

Microscopic lesions

Histologic examination of hematoxylin and eosin stained sections of synovial and subsynovial tissues, myotendon junctions and patellae of the 2 groups of intraarticularly infected lambs revealed a consistent inflammatory reaction which gradually changed in character and increased in severity. The inflammatory reaction progressed from an initial serous reaction to a fibrinopurulent inflammation and finally to

proliferation of fibrous tissue.

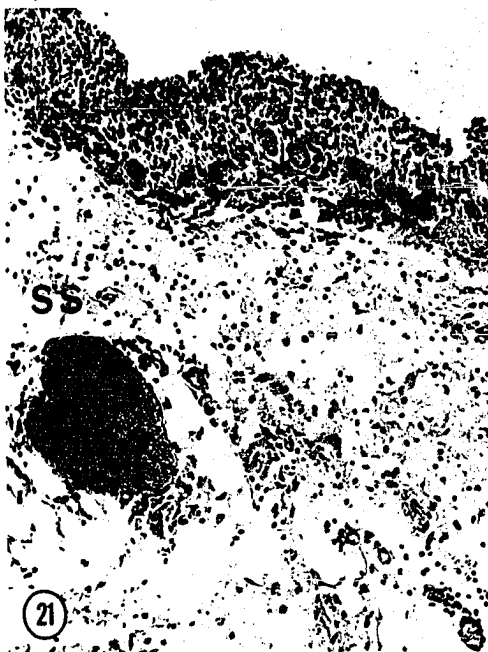
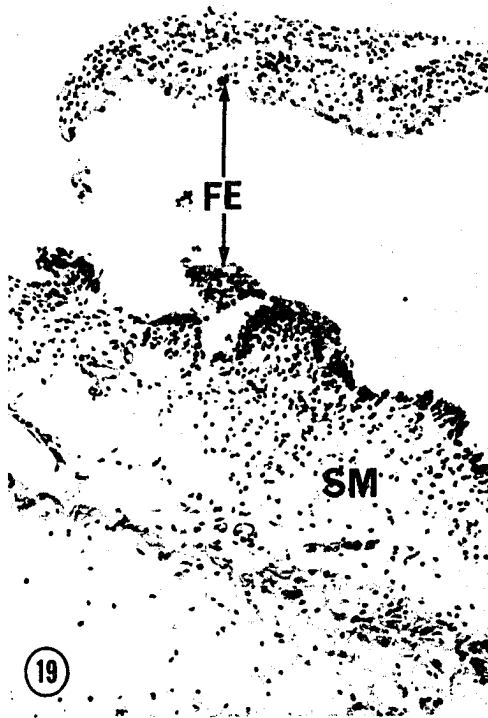
At 2-14 hours postinoculation, synovial membranes were edematous, hyperemic and infiltrated with neutrophils. A variable amount of fibrin was present on the synovial surfaces (Figure 19). This reaction was slight at 2 hours but at 14 hours the synovium was infiltrated heavily with neutrophils and overlaid with considerable fibrin. Cellular infiltration and fibrin deposition were more extensive around tendons. Many neutrophils were associated with vessels, either in their lumens or in perivascular tissues.

After 24 and 48 hours of infection, the inflammatory process was similar to that at 14 hours but synovial involvement was more extensive. Areas of the synovial membranes under the fibrinopurulent exudate were necrotic and heavily infiltrated with neutrophils (Figure 20). In addition to neutrophils, the synovial and subsynovial tissues were infiltrated with several lymphocytes. They were edematous, hyperemic and had multiple hemorrhagic foci; the viable lining cells were hyperplastic and hypertrophic with an increased cytoplasmic basophilia (Figure 21).

Except for a small amount of fibroplasia and the presence of more lymphocytes and cells of the plasmacytic series, the reactions at 72 and 96 hours postinoculation were similar to that at 48 hours. A few macrophages were present in the necrotic debris and fibroblasts had proliferated at the junction of the fibrinous exudate and synovial tissue (Figure 22).

- Figure 19. Acute serofibrinous synovitis 8 hours after intraarticular injection of the OCP agent. The edematous synovial membrane (SM) containing emigrating leukocytes (primarily neutrophils) is covered by small tags of fibrinous exudate (FE). Hematoxylin and eosin. X 125
- Figure 20. Acute fibrinopurulent synovitis of an adipose membrane 24 hours after injection of the OCP agent. The synovial lining (SL) is necrotic, hemorrhagic, infiltrated with neutrophils, and overlaid with fibrinopurulent exudate (FPE). Hematoxylin and eosin. X 32
- Figure 21. Synovium of a joint infected for 48 hours with OCP. The synovial lining (SL) and sublining (SS) layers are edematous, hyperemic, and infiltrated with neutrophils and lymphocytes. Lining cells are hyperplastic and hypertrophic. Hematoxylin and eosin. X 32
- Figure 22. Chronic fibrinopurulent inflammation and extensive necrosis of the synovium 96 hours after intraarticular injection of the OCP agent. Granulation tissue (GT) is subjacent to the fibrinopurulent exudate (FPE) and separated from it by a zone of neutrophils (N). Hematoxylin and eosin. X 50

FE - fibrinous exudate
FPE - fibrinopurulent exudate
GT - granulation tissue
N - neutrophils
SL - synovial lining
SM - synovial membrane
SS - synovial sublining



Focal hemorrhages were common in the areas of necrosis and the inflammatory cells extended to a greater degree into the periarticular tissues. Involvement of subsynovial connective tissue and muscle was limited primarily to perivascular accumulations of mononuclear cell (Figure 23). The reaction was similar 6 days postinoculation with the exception of additional fibrosis.

At 10 and 12 days after inoculation, fibrosis was more extensive with highly vascularized fibrous villi frequently projecting from the synovial surfaces (Figure 24). The newly formed fibrous tissue was hyperemic and infiltrated with inflammatory cells. These were primarily lymphocytes but also included a large number of mature and immature plasmacytes (Figure 25). The same type cells had accumulated in the subsynovial perivascular tissues. Far fewer neutrophils were present than in joints of lambs killed earlier; those present were largely confined to synovium still covered with fibrin and within the fibrin sheets. Large sheets of fibrin, firmly attached to the synovium by granulation tissue, remained over large areas of the synovial tissues (Figure 26).

After 21 and 24 days of infection, granulation tissue had replaced much of the fibrinopurulent exudate with the formation of large fibrous villi (Figure 27). Synovial surfaces were recovered by intact lining cells. The fibrous tissue contained numerous focal accumulations of lymphocytes with a diffuse distribution of plasmacytes (Figure 27).

Figure 23. Lymphocytes in periarticular muscle 72 hours after injection of the OCP agent. Hematoxylin and eosin. X 320

Figure 24. Fibrotic synovium of a lamb 10 days after receiving the OCP inoculum. Vascularized fibrous villi, infiltrated with chronic inflammatory cells, project into the joint space. Hematoxylin and eosin. X 32

Figure 25. Higher magnification of the synovium in Figure 24. Interspersed with the fibrous tissue are lymphocytes, plasmacytes, neutrophils, and synovial cells. Hematoxylin and eosin. X 320

Figure 26. Partially organized fibrinous exudate in the joint of a lamb 10 days postinoculation. Fibrin (F) is separated from the granulation tissue (GT) by a few neutrophils (N). Hematoxylin and eosin. X 50

F - fibrin
GT - granulation tissue
H - hemorrhage
JS - joint space
N - neutrophils

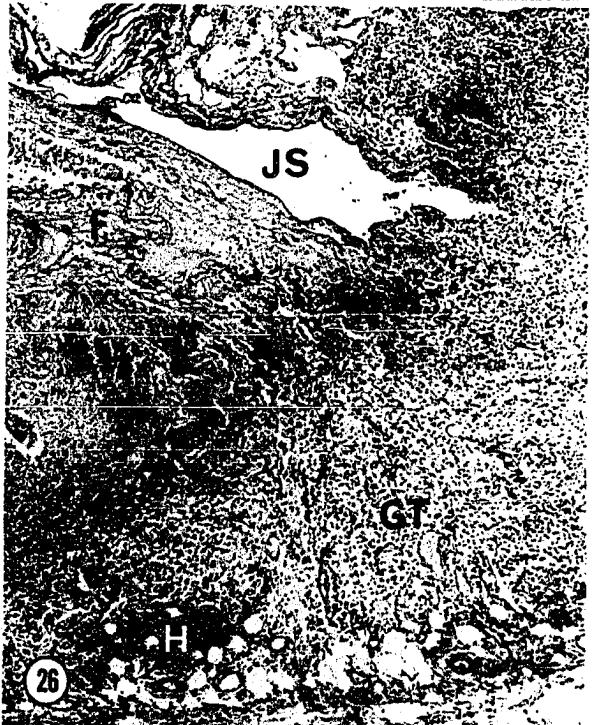
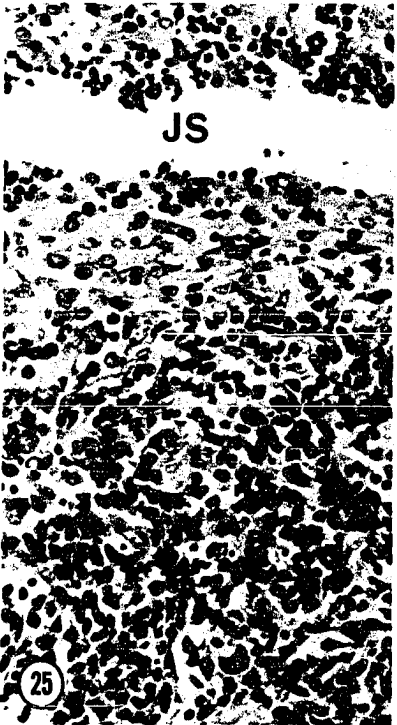
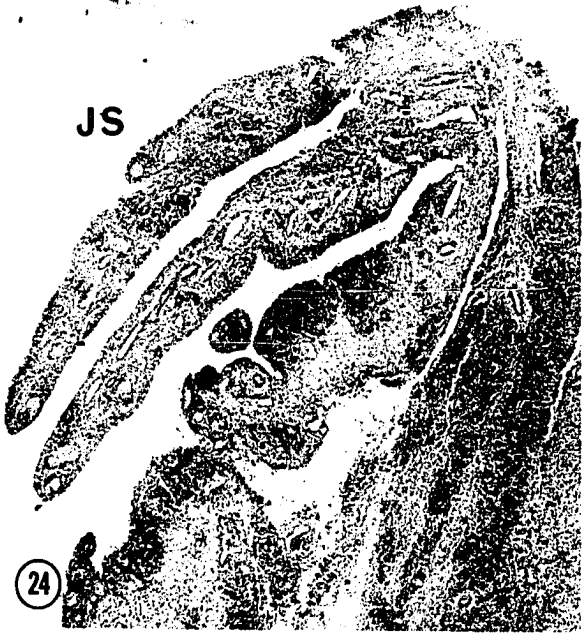
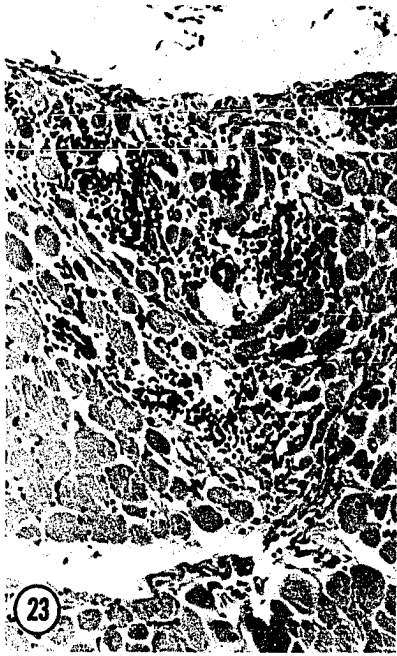
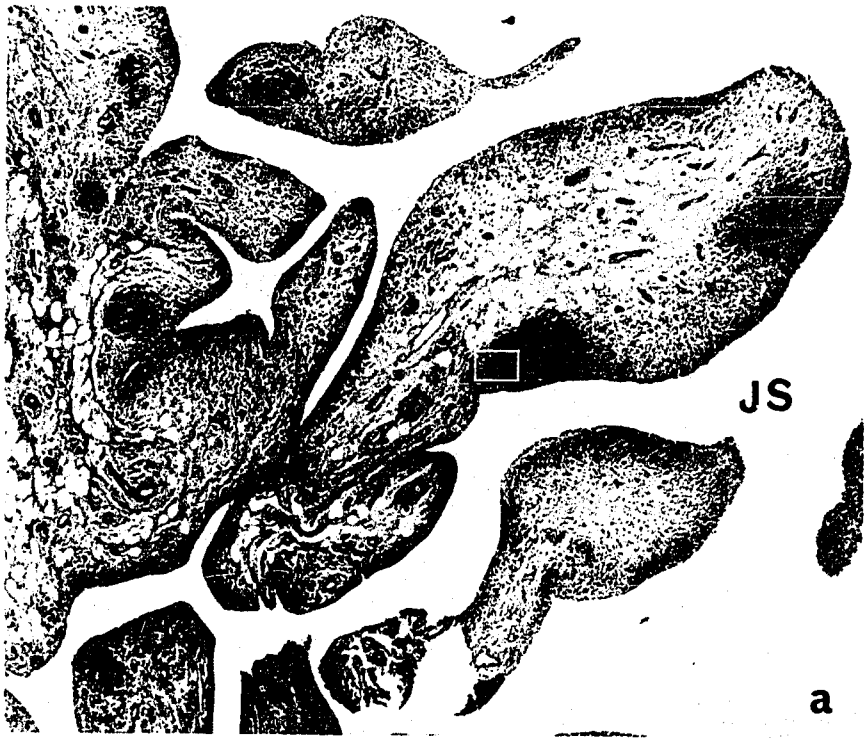


Figure 27. Chronic synovitis 21 days after intraarticular injection of the OCP agent. (a) Focal accumulations of lymphocytes and disperse plasmacyte infiltration of hyperplastic synovial villi. (b) Higher magnification of area indicated in (a) containing the edge of a lymphoid nodule (L) and surrounding plasmacytes (PC). Hematoxylin and eosin. a. X 32; b. X 500

JS - joint space
L - lymphocytes
PC - plasmacytes



Noninoculated joints of all lambs were normal upon histologic examination. Joints of lambs inoculated with egg yolk sac and killed 24 hours or earlier after inoculation were edematous, hyperemic and infiltrated with a few neutrophils. After 48 hours, the egg-yolk-sac-inoculated joints appeared normal.

Smears of synovial fluid from infected joints contained numerous neutrophils and macrophages, the latter frequently with phagocytized material including degenerate neutrophils in their cytoplasm. Neutrophils predominated in most joints but were fewer in number after 6 days of infection. Smears from joints inoculated with egg yolk sac were similar but fewer cells were present. Few cells were present in smears from noninoculated joints.

With Giemsa staining, blue cytoplasmic inclusions were found in sections of synovial membranes and smears of synovial fluid from joints inoculated with either the OCP agent or egg yolk sac. Most were believed to represent phagocytized cellular debris; positive identification of chlamydial inclusions could not be made.

Articular changes in naturally and intravenously infected lambs

Pathologic changes in the joints of naturally infected lambs were similar to those found in joints inoculated intra-articularly at least 10 days previously. The femorotibial, tarsal, and carpal joints were most severely affected. Major changes were infiltration of the synovial tissues with plasma-

cytes, lymphocytes, and neutrophils and proliferation of fibrous connective tissues. In 2 lambs, partially organized fibrinopurulent exudate covered zones of necrotic synovial tissue and in all lambs, the joint capsules were thickened as a result of excessive fibrous tissue. A few small fibrous fronds extended into the joint cavities.

The synovial membranes were hyperemic and edematous with a few petechia. In some joints, the inflammatory reaction extended into periarticular muscle and fascia. Copious synovia containing flakes of fibrin, neutrophils, and macrophages were present in the joints. Gross and histologic examination of articular cartilage and epiphyseal bone revealed no pathologic alteration.

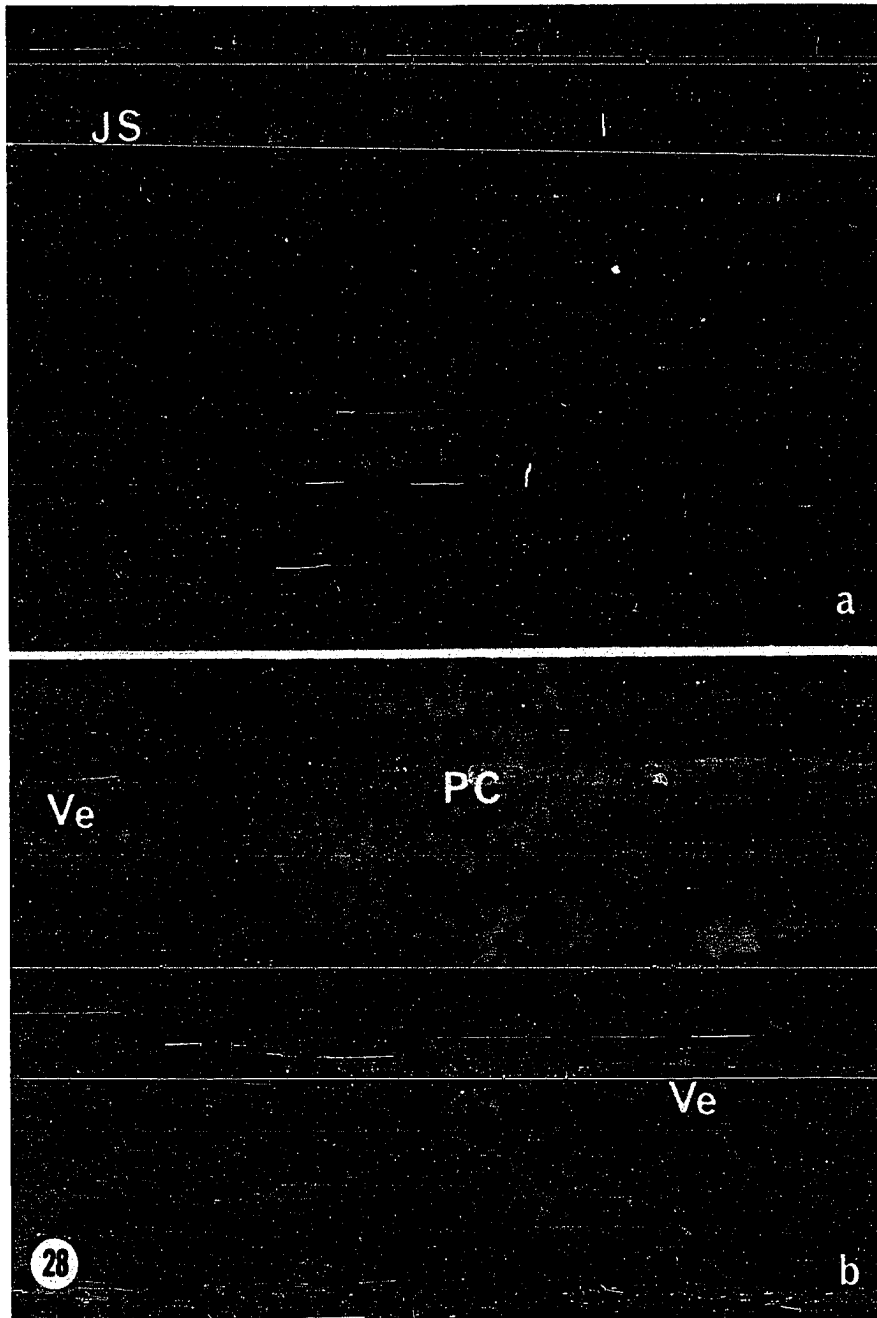
Alterations in the joints of the 2 lambs infected intravenously were minimal; they were similar to those found in the 2-hour intraarticularly infected lamb, except neutrophils were fewer in number and the lining cells of one lamb were hyperplastic. The synovial membranes were edematous, hyperemic and infiltrated with a few neutrophils and lymphoid cells. Neutrophils and small flakes of fibrin were present in the synovial fluid.

Histochemistry

Acridine orange The nuclei of cells within the synovium of all lambs fluoresced yellowish-green (Figure 28). Except for an increased number of cells in infected joints,

Figure 28. OCP infected synovial membranes of the femoro-tibial joints of yearling lambs stained with acridine orange. (a) After 8 hours of infection only green nuclear fluorescence is visible; whereas, (b) after 21 days of infection several plasmacytes (PC) with orange cytoplasmic fluorescence are seen adjacent to small vessels (Ve). a. X 125; b. X 320

JS - joint space
PC - plasmacytes
Ve - vessels

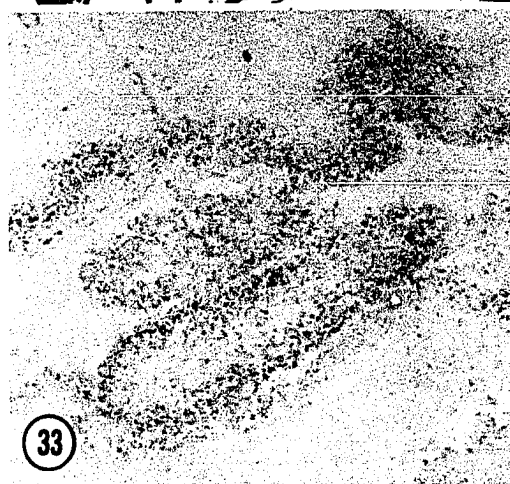
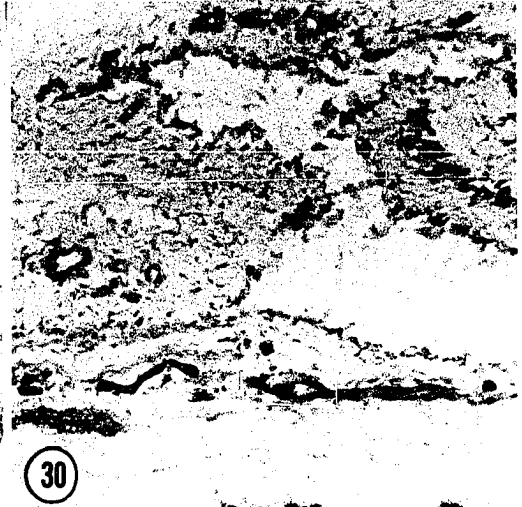
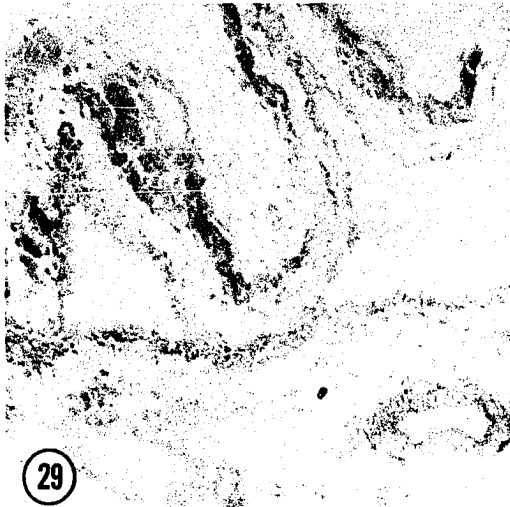


difference was not apparent between nuclear fluorescence of infected and noninfected joints. Cytoplasmic fluorescence was seen only in joints infected for 96 hours. A few cells within the synovium of the lamb killed 96 hours after inoculation had orange fluorescent foci in the cytoplasm. At 10-21 days postinoculation, the entire cytoplasm of numerous cells in the synovial and subsynovial tissues fluoresced bright orange (Figure 28). Cells of this type were widely distributed but most were in a perivascular location. Their plasma-cyte morphology was confirmed by staining with Giemsa subsequent to acridine orange.

Alcian blue-periodic acid Schiff and oil red O The cytoplasm of normal synovial cells stained faintly with the periodic acid-Schiff reagent and the intercellular matrix of the synovial lining stained lightly with Alcian blue. Periodic acid-Schiff staining outlined the limiting outer margin of the synovium formed by cytoplasmic processes of synovial lining cells. Except for disruption of the synovium and the addition of inflammatory cells, the staining reaction was not altered by infection. Diastase had no effect on staining. Lipid, which stained with oil red O, was abundant in all infected and control joints.

Acid phosphatase In noninfected joints, there was light acid phosphatase activity in synovial lining cells and vascular endothelium; other cells stained only faintly or not at all (Figure 29). In joints infected with the OCP agent,

- Figure 29. Synovium of a normal lamb stained by Gomori's lead method for acid phosphatase activity. Light staining is present in the synovial lining cells and vessels. X 125
- Figure 30. Synovium of a normal lamb stained by the Gomori-Takamatsu method for localizing alkaline phosphatase activity. Enzymatic activity is limited to vessels. X 125
- Figure 31. Normal synovial membrane stained by the calcium method of Padykula and Herman for adenosine triphosphatase activity. Enzymatic activity is localized in lining cells and vessels. X 320
- Figure 32. Normal synovium of a yearling lamb with formazan pigment, indicating activity of reduced diphosphopyridine nucleotide diaphorase in the synovial lining cells and vascular endothelium. Stained by the method of Scarpelli, as modified by Barka and Anderson, with the tetrazolium salt, Nitro BT. X 125
- Figure 33. Light deposition of formazan pigment in the lining cells of noninfected synovium after staining for succinic dehydrogenase activity. Stained by the tetrazolium method of Nachlas, as modified by Barka and Anderson, utilizing Nitro BT as the hydrogen acceptor. X 125
- Figure 34. Synovium of noninfected joint with glucose-6-phosphate dehydrogenase activity localized in the synovial lining cells and vascular endothelium. Stained by the tetrazolium method of Nachlas, as modified by Barka and Anderson, with Nitro BT as the hydrogen acceptor. X 125



inflammatory cells (primarily neutrophils) in the exudate stained intensely in addition to the synovial structures. At 10 and 21 days postinoculation, new fibrous tissue cells were also moderately stained. Substrate-deficient controls were negative.

Alkaline phosphatase This enzyme was limited to vessels and neutrophils. Staining of the synovium of infected joints paralleled the presence of neutrophils in the exudate; thus, staining was most intense at 48 to 96 hours postinoculation. A few neutrophils in the synovium of joints injected with the egg-yolk-sac inoculum and killed at 16 and 24 hours had enzymatic activity; otherwise, only vessels of the non-infected joints were stained (Figure 30). Precipitate was not present in the substrate-deficient controls.

Adenosine triphosphatase In noninfected synovium, cells of the surface layer and vessels stained intensely; fibrocytes in the subsynovial tissues were lightly stained (Figure 31). In addition to normal structures, inflammatory cells and fibroblasts of infected joints contained adenosine triphosphatase activity.

Reduced diphosphopyridine nucleotide diaphorase
Activity of this enzyme was heaviest in the synovial lining cells with light activity in endothelial cells (Figure 32). Little difference was detected between infected and noninfected synovium, except the staining of more cells in the subsynovial tissue of infected joints. All staining appeared

to be confined to the cytoplasm of cell.

Succinic dehydrogenase Succinic dehydrogenase activity was very light in noninfected synovium and confined to the synovial lining cells (Figure 33). Similar results were found in infected joints prior to 72 hours postinoculation. Subsynovial tissues of the lambs killed 72 hours postinoculation contained several cells, probably of the plasmacytic series, with enzymatic activity.

Glucose-6-phosphate dehydrogenase Activity of this enzyme was confined to the synovial lining cells and vascular endothelium of noninfected joints (Figure 34) and of joints infected for less than 10 days. After 10 days, enzymatic activity was also found in fibroblasts. Intensity of staining varied greatly between individual lambs. Substrate-deficient controls were negative.

Immunohistochemistry

When stained by the fluorescent antibody technique, intracytoplasmic fluorescent granules were found in synovial membrane sections of all infected joints of the yearling lambs. Many infected joints had cells in the synovial fluid which contained fluorescent cytoplasmic granules. Prior to 72 hours postinoculation, only an occasional cell fluoresced; whereas, after 72 hours several cells in the synovial tissues and exudate fluoresced a bright green. Blocking the reaction with nonconjugated antichlamydial globulin did not inhibit all

fluorescence; but did appear to decrease the number of fluorescent cells. When the tissues were stained with Giemsa, subsequent to fluorescent antibody staining, many of the fluorescent cells were found to be eosinophils. Eosinophils were most numerous in lambs killed 72 and 96 hours postinoculation. Neither fluorescent cells or eosinophils were found in noninoculated joints.

With the enzyme antibody technique, there was diffuse staining of both infected and noninfected synovial tissue. This was true even without addition of the enzyme antibody conjugate. Tissue sections did not stain when benzidine was omitted from the incubating solution.

Electron microscopy¹

Regional variations in the morphology of normal synovial membranes were recognized by electron microscopy. In some regions, which probably correspond to the fibrous membrane of light microscopy, the synovium was characterized by widely spaced cells indistinguishable from cells in the subsynovial fibrous tissue (Figures 35 and 36). Lining cells were stellate to slightly elongated; some had long cytoplasmic processes and surface folds. Elongated cells were orientated with their long axis parallel to the synovial surface. Nuclei had multiple indentations and diffuse clumps of chromatin.

¹Terminology of ultrastructure is according to Fawcett (1966).

Figure 35. Electron micrograph of fibrous synovium from the femorotibial joint of a normal lamb. Lining cells resembling fibroblasts rest on dense collagen (Co) and are surrounded by a granular intercellular matrix (IM) containing bundles of fine, 110 A, fibrils (FF). Note the dense surface matrix (DSM). Uranyl acetate and lead citrate. X 4,400

Co - collagen
DSM - dense surface matrix
FF - fine fibrils
IM - intercellular matrix
JS - joint space

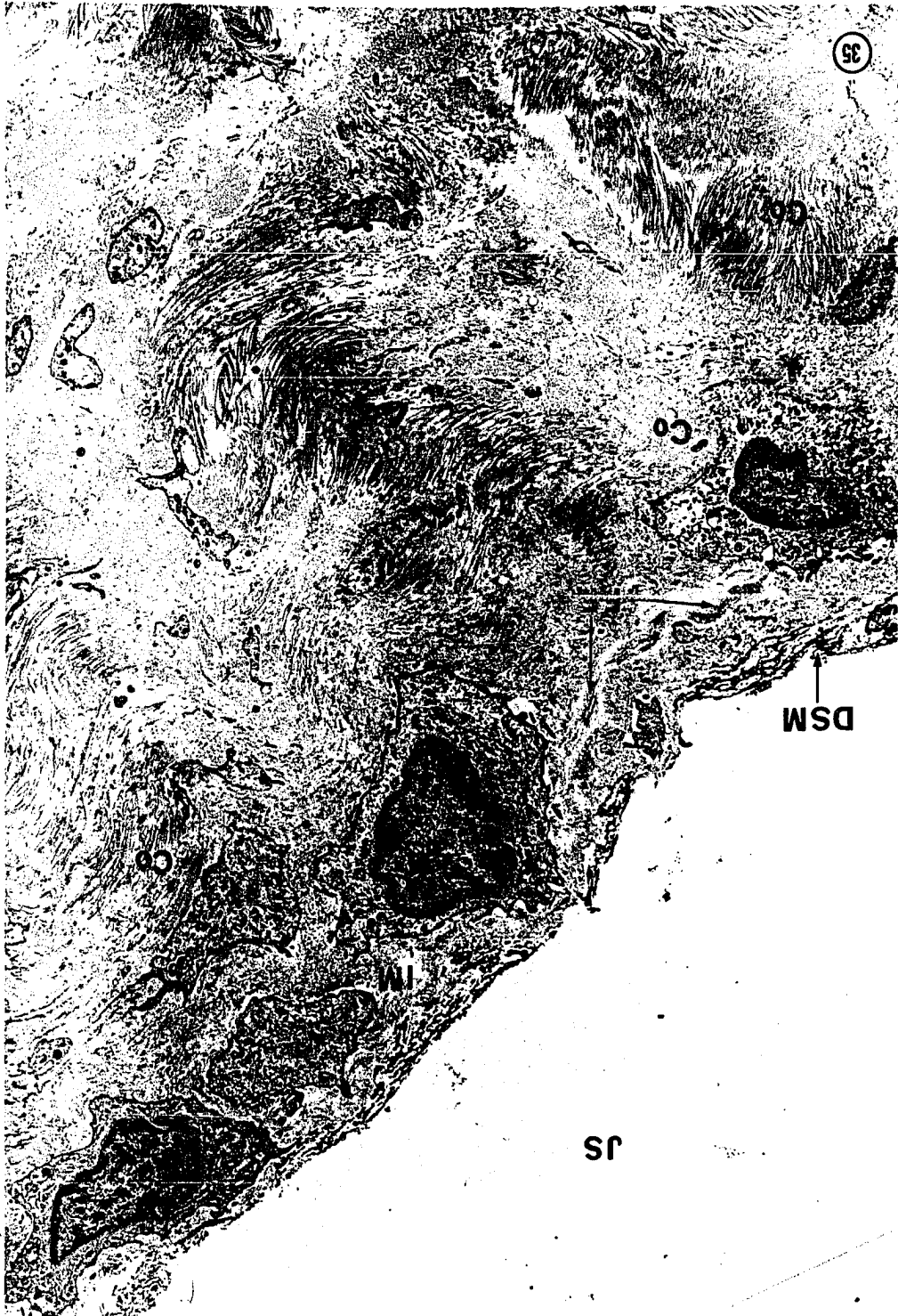


Figure 36. Electron micrograph of a lining cell from fibrous synovium of a normal lamb. The cell is characterized by short saccules of granular endoplasmic reticulum (GER), a Golgi apparatus (GA), several small vesicles (SV), mitochondria (M), numerous fine, 110 A, fibrils (FF) especially in the perinuclear region, and larger, 255 A, fibrils (arrows). Uranyl acetate and lead citrate. X 18,000

Co - collagen
DSM - dense surface matrix
FF - fine fibrils, intracellular and extracellular
GA - Golgi apparatus
GER - granular endoplasmic reticulum
IM - intercellular matrix
JS - joint space
M - mitochondria
NP - nuclear pores
SV - small vesicles
Arrows - large intracellular fibrils



Cells of this type had a moderate amount of granular endoplasmic reticulum in the form of short saccules which often were dilated. Several mitochondria were present and a Golgi apparatus was seen in a few of the cells. Numerous fine fibrils (100-125 A) were scattered through the cytoplasm with the heaviest concentration in the perinuclear zone. Larger fibrils (225-250 A) were seen in some cells. Several small vesicles were scattered throughout the cytoplasm. The intercellular matrix consisted of a fine granular substance containing fine fibrils similar to those in the cytoplasm of lining cells. The matrix usually completely surrounded the lining cells; in some areas the synovial surface was composed of a more opaque material than that surrounding the lining cells. The lining layer of synovium rested on dense collagenous tissue. Collagenic fibers occasionally extended into the intercellular matrix of the lining layer but were not observed to reach the synovial surface.

Other regions of synovial membranes were composed of cells similar in characteristics to Barland's A and B types. Mast cells were found rarely (Figure 37). Cell concentration varied from areas with large intercellular spaces to areas with 1-3 rows of closely approximated cells. In some areas, the luminal surface was made up of cytoplasmic processes and intercellular matrix (Figure 38); however, in most areas the cells were directly exposed to the joint space (Figure 39). Intercellular matrix was similar to that in the fibrous type

Figure 37. Mast cell from the synovial membrane of a normal lamb joint. Uranyl acetate and lead citrate. X 18,000

Co - collagen



Figure 38. Normal areolar synovium sparsely populated with type A (A) and type B (B) lining cells. The surface is covered by cytoplasmic processes (CP) of lining cells and intercellular matrix (IM). An arteriole, containing an erythrocyte (RBC), is present at upper left. Uranyl acetate and lead citrate. X 4,400

A - type A synovial lining cell
B - type B synovial lining cell
Co - collagen
CP - cytoplasmic processes of lining cells
EC - endothelial cell
IM - intercellular matrix
JS - joint space
Mus - smooth muscle
P - pericyte
RBC - erythrocyte

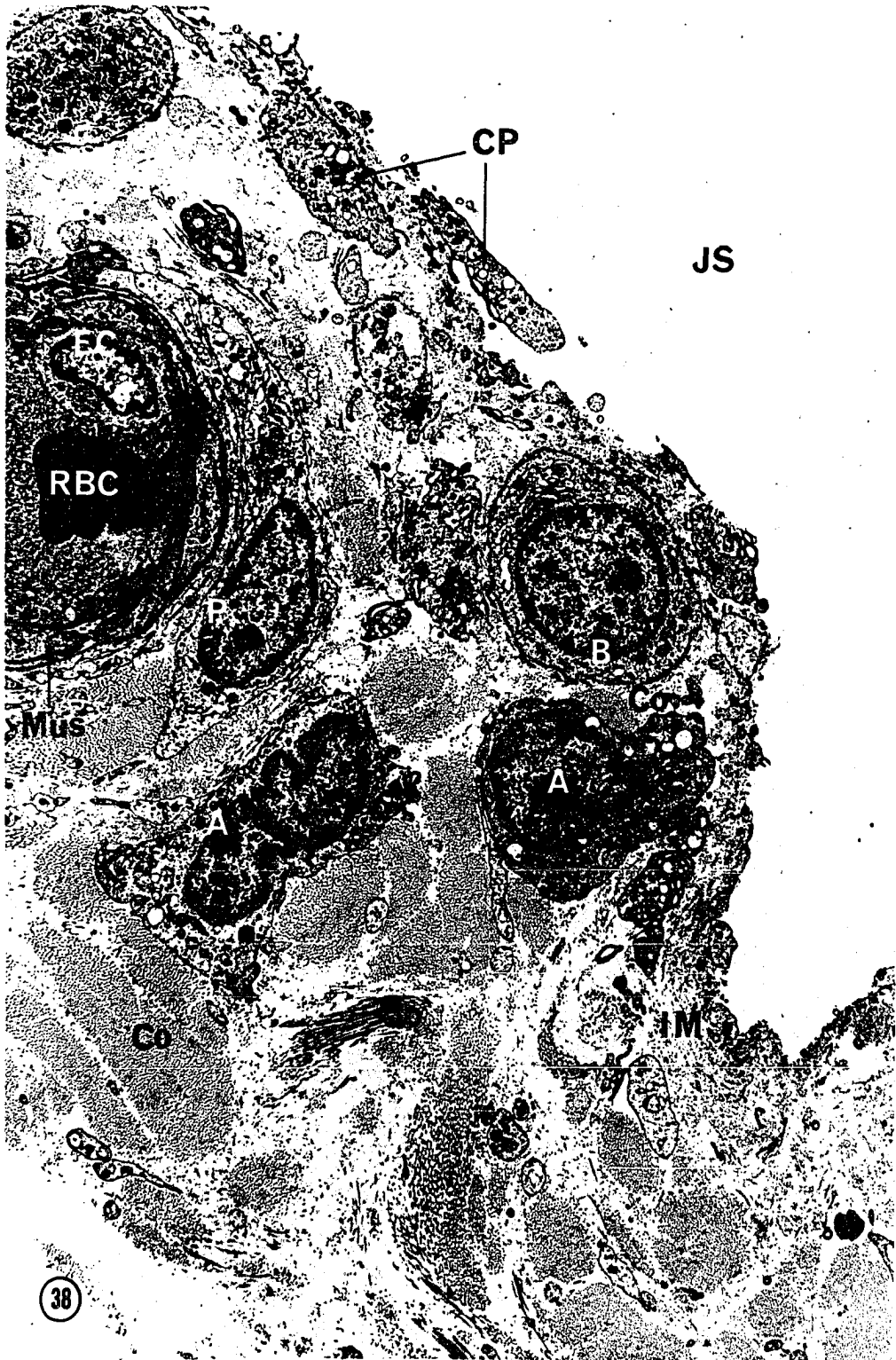
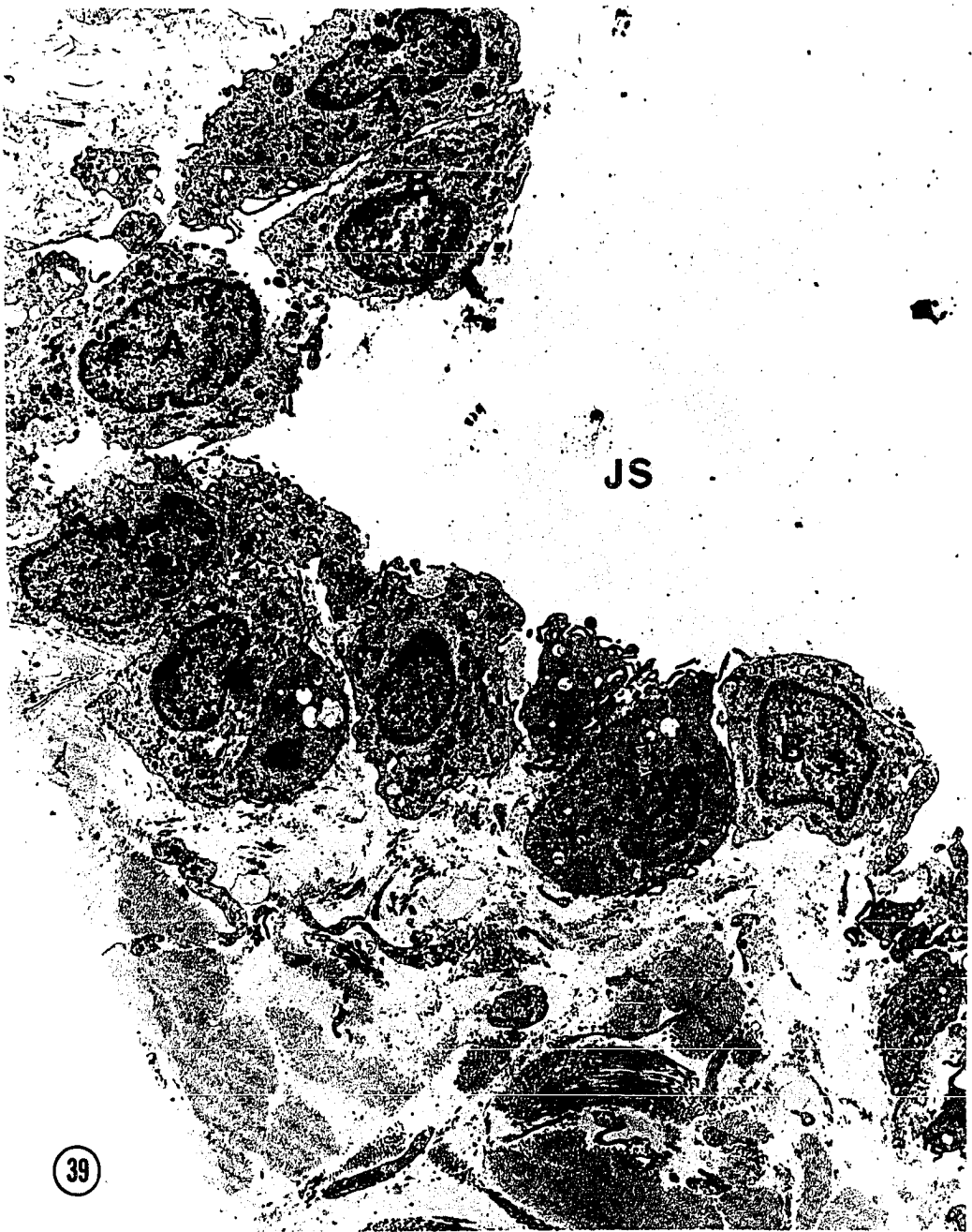


Figure 39. Normal areolar synovium composed of closely packed type A (A) and type B (B) lining cells. Uranyl acetate and lead citrate. X 4,400

A - type A synovial lining cells
B - type B synovial lining cells
Co - collagen
JS - joint space



synovium. This variety of synovium was found over highly vascular fibrous tissue and probably corresponds to the areolar membrane of light microscopy.

Type A cells (Figures 38, 39 and 40) were characterized by membrane-bound granules of variable electron density and size (up to 1 micron in diameter), vesicles of similar size which may represent pinocytotic vesicles, and numerous small vesicles which resembled fuzzy and smooth type micropinocytotic vesicles. The small and large vesicles frequently contained a moderately dense substance. Similar small vesicles were present in the Golgi apparatus which was seen in many type A cells. A few short saccules of granular endoplasmic reticulum and many free ribosomes were present in most cells. A few cells with free ribosomes but few or no granules, but otherwise characteristic of the type A cell, were found. Cell outlines were inconsistent, usually with long cytoplasmic processes and numerous surface folds which occasionally appeared to form pinocytotic vesicles. The nuclei were irregular in outline, frequently with margined clumps of chromatin. Most cells of this type were located in the surface layer of synovial lining cells.

Type B cells (Figures 38, 39 and 41) were characterized by a large amount of granular endoplasmic reticulum which was usually in the form of relatively short saccules of flattened cisternae. A few large vesicles and electron-opaque granules, similar to those in the type A cells were present. Many type

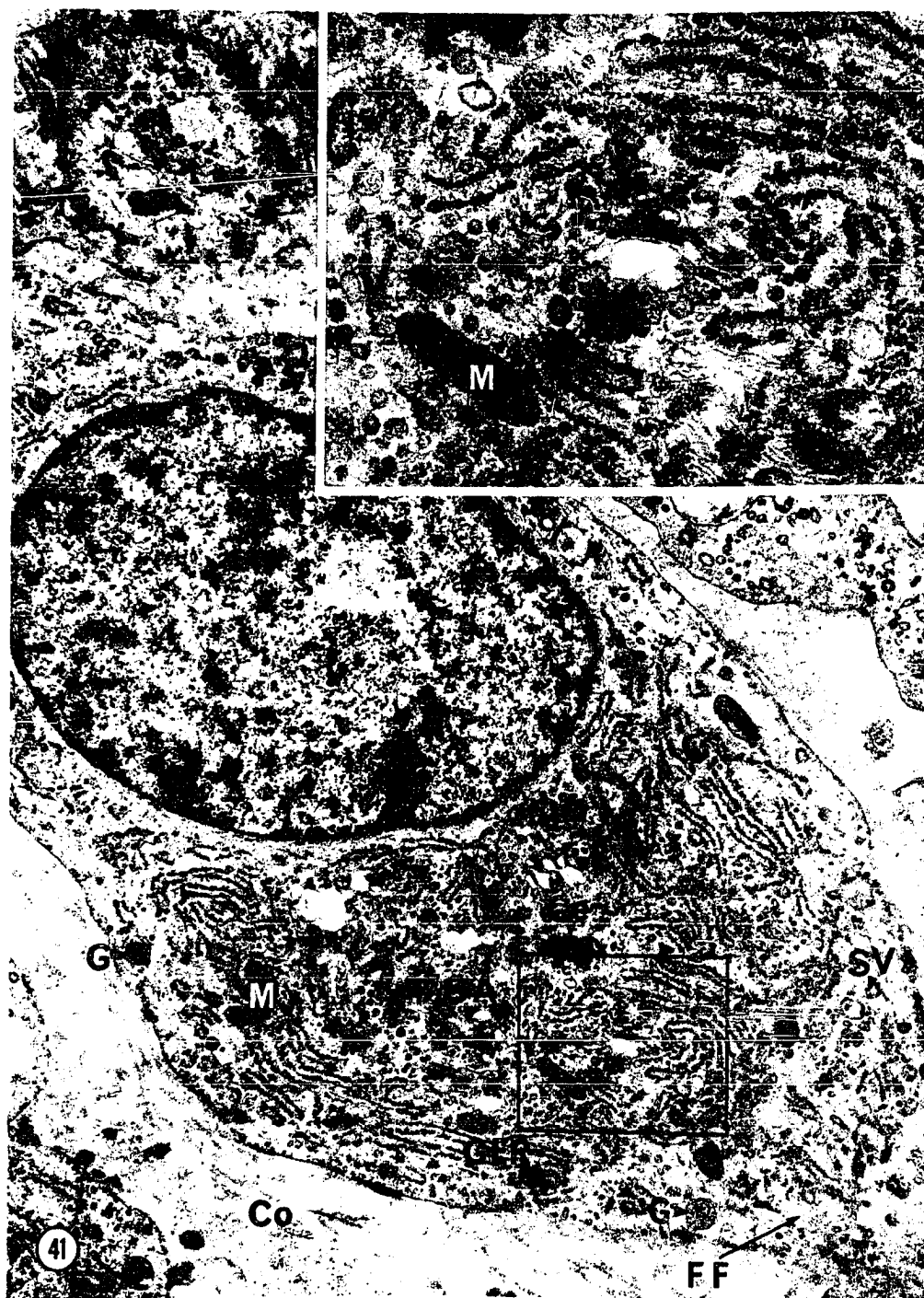
Figure 40. Type A synovial lining cell from a normal joint. This type cell is characterized by large granules (G), numerous small vesicles (SV) which may be either micropinocytotic vesicles or vesicles from the Golgi apparatus, and large vesicles (LV) which may represent pinocytotic vesicles formed by surface folds (SF) such as those at the left. Inset: higher magnification of area in the square. Note the limiting membrane (arrows) and varying internal structure and density of the large granules. Uranyl acetate and lead citrate.
X 14,000; inset X 41,000

Co - collagen
FF - fine fibrils
G - granules
LV - large vesicles
M - mitochondria
SF - surface folds
SV - small vesicles
Arrows - limiting membrane of granules



Figure 41. Electron micrograph of a type B synovial lining cell from a normal joint. The cell is characterized by regular outline, a network of granular endoplasmic reticulum (GER), several small vesicles (SV), a few granules (G), and an extensive Golgi apparatus (GA), a portion of which is shown in the inset of marked area. Golgi apparatus is composed of flattened and dilated saccules surrounded by numerous small vesicles many of which contain a moderately dense substance. Uranyl acetate and lead citrate.
X 14,000; inset X 41,000

Ce - centriole
Co - collagen
FF - fine fibrils
G - granules
GA - Golgi apparatus
GER - granular endoplasmic reticulum
M - mitochondria
SV - small vesicles



B cells had a relatively extensive Golgi apparatus consisting of flattened saccules and many small vesicles similar to those in the type A cells. Usually, some of the saccules of the Golgi complex were dilated. Nuclei were uniform in outline with chromatin clumps slightly more disperse than in the type A cell. Cellular outlines were more regular than type A cells; however, small cytoplasmic projections and surface folds were present on some cells. Both type A and B cells had fine intracytoplasmic fibrils similar to those in the cells of the fibrous membrane.

Electron microscopic examination of synovial membranes and synovial fluid of experimentally and naturally infected lambs revealed an inflammatory reaction which agreed with and complemented observations made with the light microscope. The synovial tissues were infiltrated with numerous inflammatory cells, of which the neutrophil was most common. Fibrin was abundant. In late infections, lymphocytes and plasma cells were prevalent along with fibroblasts.

In lambs killed before 14 hours of infection, synovial membranes were normal except for infiltration of inflammatory cells and localized degeneration of lining cells (Figure 42). After this time, there were large areas where the synovium was necrotic and disrupted or replaced by a fibrinocellular exudate (Figure 43).

By 96 hours postinoculation and afterward, the type B lining cells were increased in number and contained more

Figure 42. Acute serous synovitis of a lamb 8 hours after intraarticular injection of the OCP agent. Degenerate lining cells (DLC) and edema (upper) are adjacent to an area of normal type A (A) and type B (B) lining cells between which emigrating leukocytes (L, N) are found. Uranyl acetate and lead citrate. X 4,100

A - type A synovial lining cells
B - type B synovial lining cells
DLC - degenerate lining cells
JS - joint space
L - lymphocyte
N - neutrophils

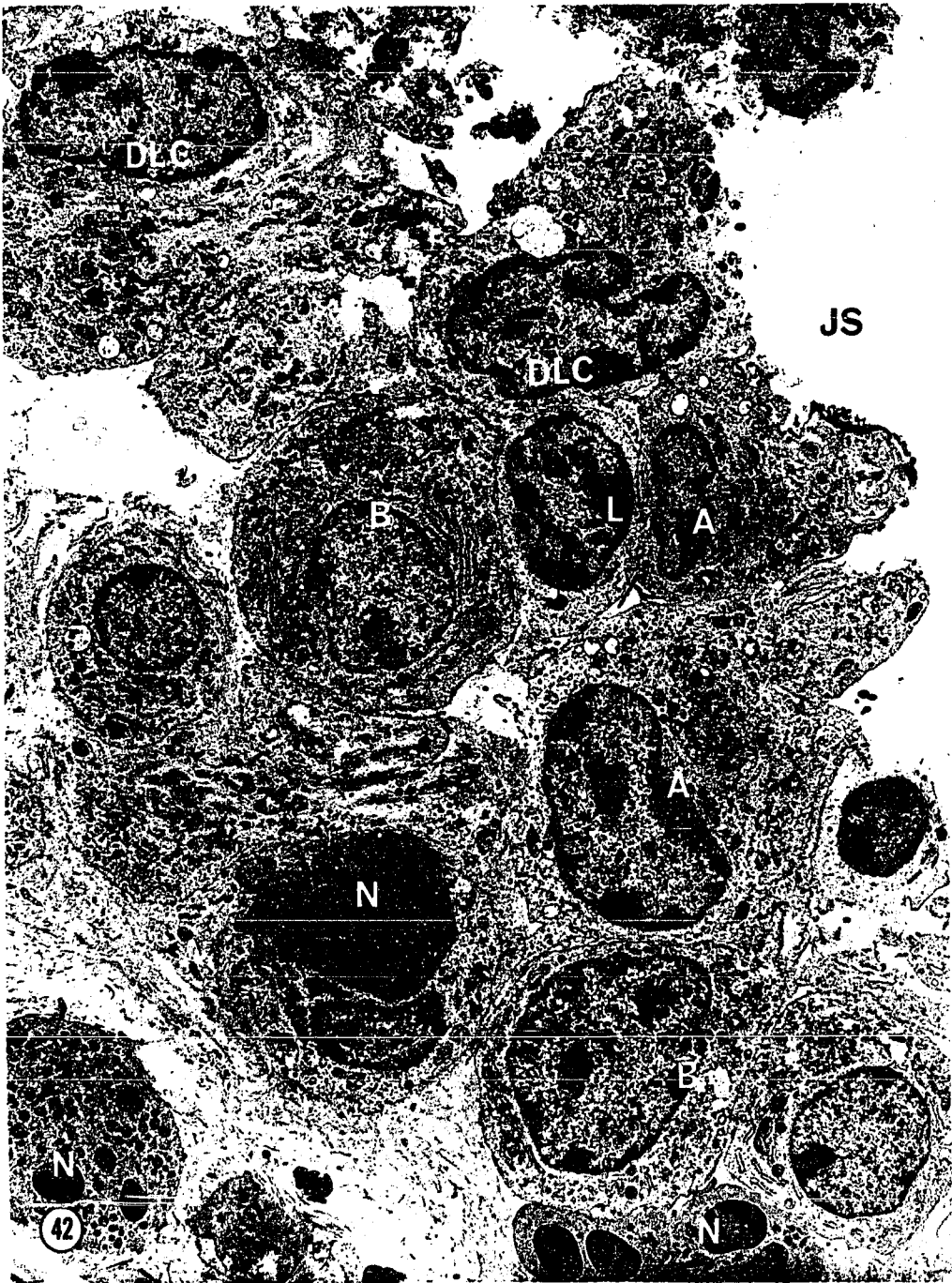
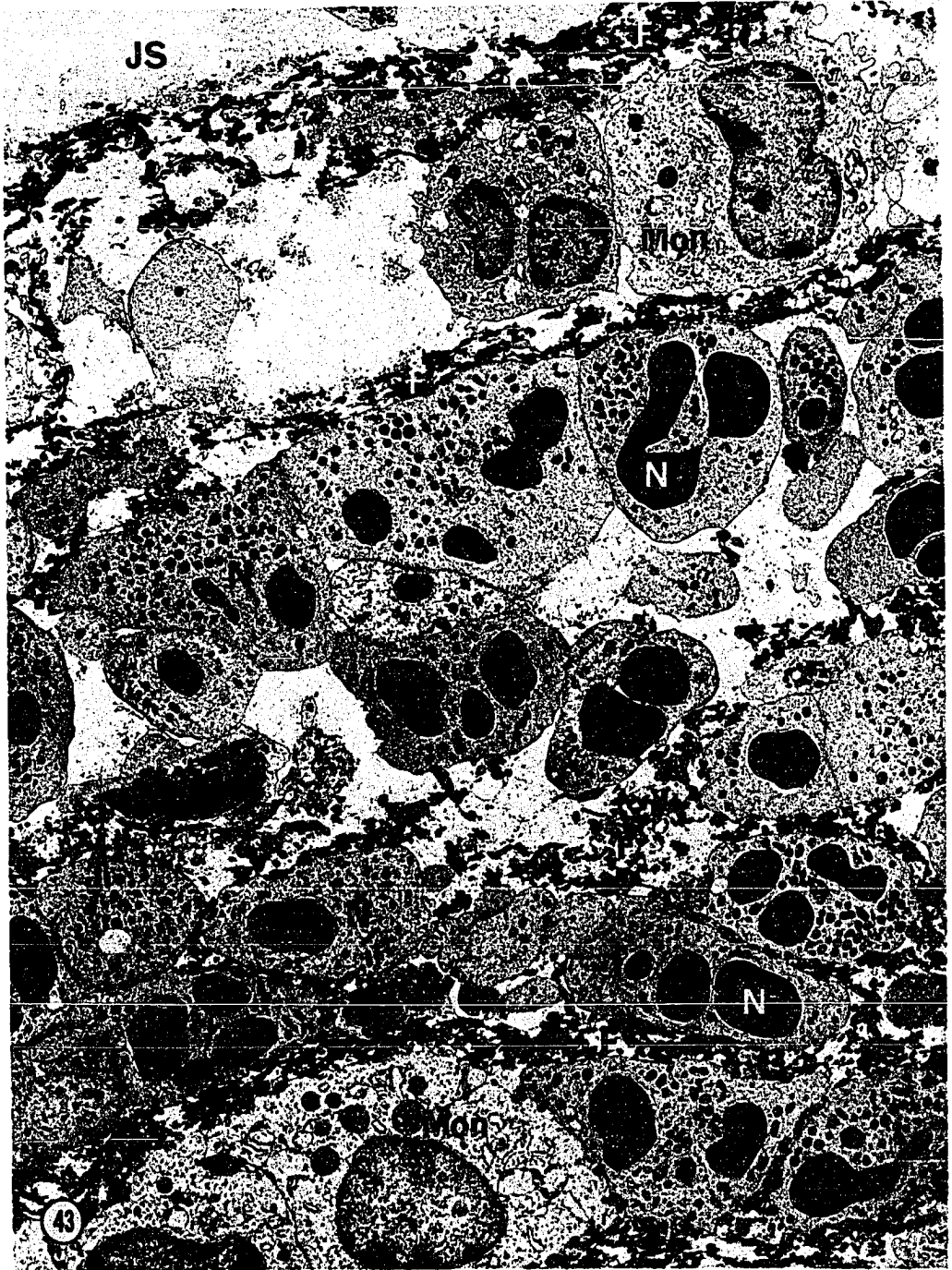


Figure 43. Acute diffuse fibrinopurulent synovitis from the joint of a lamb 48 hours after injection of the OCP agent. Note the ruptured neutrophil with free lysosomal granules (arrow) and cells with the appearance of monocytes (Mon). Uranyl acetate and lead citrate. X 4,400

F - fibrin
JS - joint space
Mon - monocytes
N - neutrophils
Arrow - lysosomal granules free in
exudate



granular endoplasmic reticulum than cells of the same type from normal joints (Figures 44 and 45). In some areas, this was the only type of cells found in the synovial lining; however, they were more frequently found in the subsurface layer with type A cells and their cytoplasmic processes comprising the surface layer of the synovium. Other cells in the surface layer possessed characteristics of both A and B type cells; i.e., endoplasmic reticulum was concentrated in the cytoplasm distal to the joint space; whereas, granules and vacuoles were adjacent to the joint space (Figure 46). This dual morphologic characteristic or polarization of lining cells was more pronounced in joints infected for a longer period of time.

By 21 days of infection, many elongated lining cells were orientated with their long axis perpendicular to the synovial surface. Several of these cells were polarized with an extensive network of granular endoplasmic reticulum surrounding the nucleus at the base and large granules and vacuoles in the apex at or near the synovial surface (Figures 47 and 48). Such cells usually had a regular outline at the base and many surface folds on the portion exposed to the joint space. In addition to polarized cells, type A cells were present at the synovial surface, many of which contained membrane-bound granules up to 2.5 microns in diameter and of variable electron density (Figure 49). Type B cells predominated in the subsurface layer of lining cells.

Figure 44. Synovial lining 96 hours after injection of the OCP agent. Hypertrophic type B cells (B) containing extensive networks of rough endoplasmic reticulum are surrounded by excessive intercellular matrix (IM) in the subsurface layer of the synovium. Irregular profiles of type A cells (A) and their cytoplasmic processes (CP) line the synovial surface. Uranyl acetate and lead citrate. X 4,400

A - type A synovial lining cells
B - type B synovial lining cells
CP - cytoplasmic processes of type A cells
IM - intercellular matrix
JS - joint space
N - neutrophil
RBC - erythrocyte

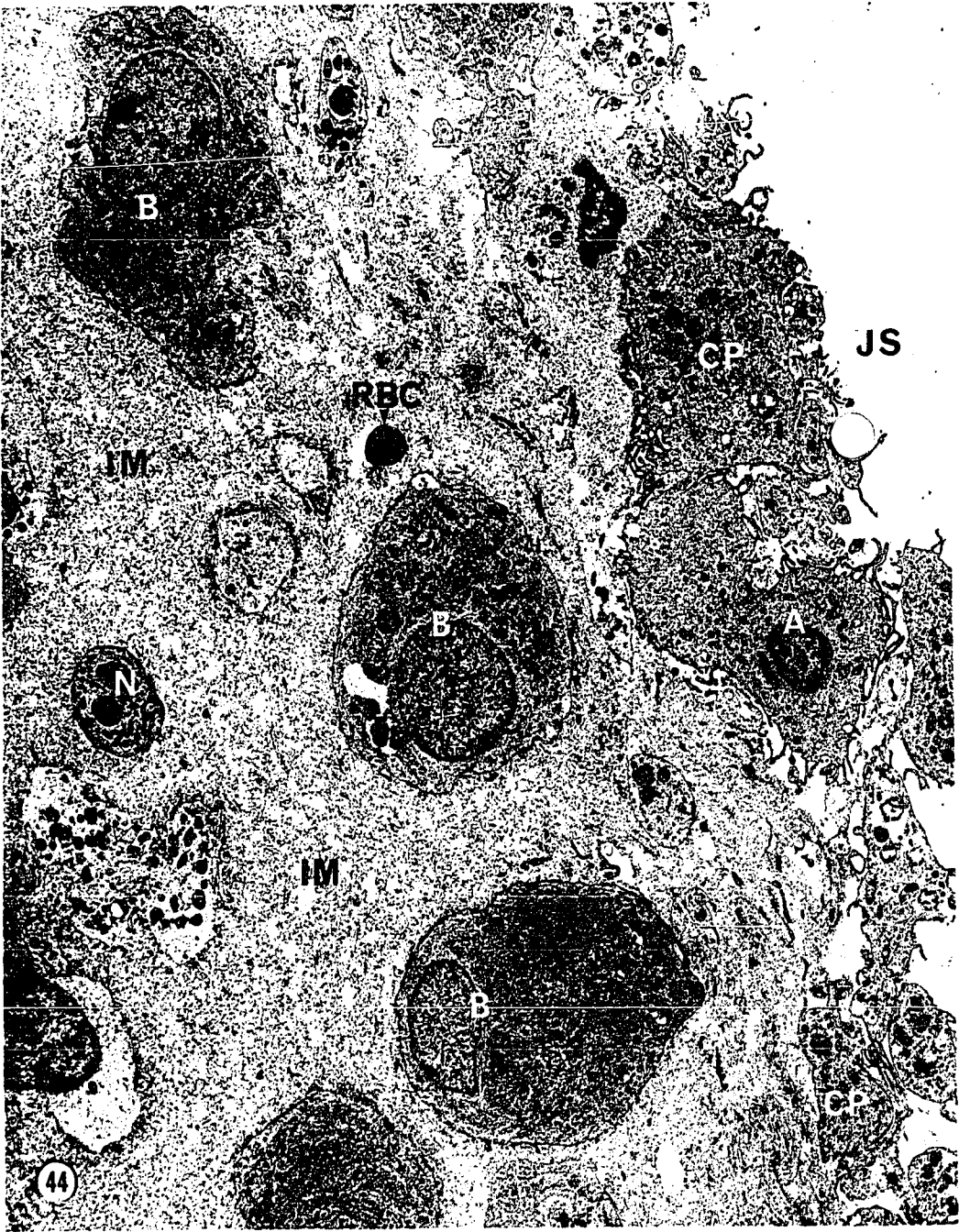


Figure 45. Hypertrophic type B synovial lining cell 96 hours after intraarticular injection of the OCP agent. Note the extensive network of granular endoplasmic reticulum (GER) and Golgi apparatus (GA) composed of dilated saccules surrounded by numerous vesicles and granules of variable size and electron density. Larger granules (G) are scattered through the cytoplasm. Uranyl acetate and lead citrate.
X 24,000

G - large granules
GA - Golgi apparatus
GER - granular endoplasmic reticulum
LG - lipid globules
M - mitochondria

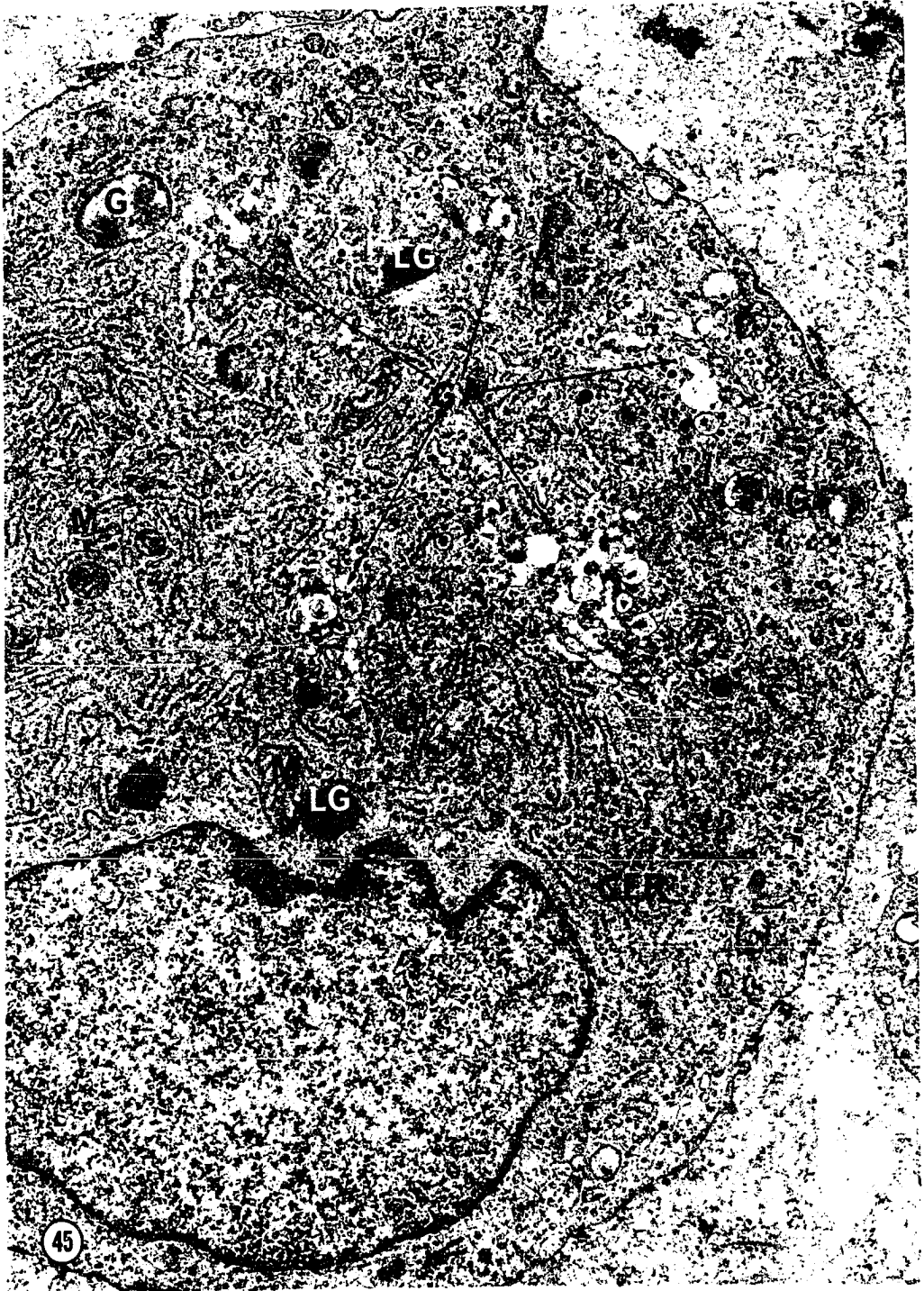


Figure 46. A synovial lining cell 96 hours after intra-articular injection of the OCP agent. Adjacent to the joint space (JS) are granules (G) and surface folds (SF), characteristic of type A lining cells; whereas, distal to the joint space is granular endoplasmic reticulum (GER), characteristic of type B lining cells. Uranyl acetate and lead citrate. X 9,600

G - granules
GA - Golgi apparatus
GER - granular endoplasmic reticulum
JS - joint space
SF - surface folds

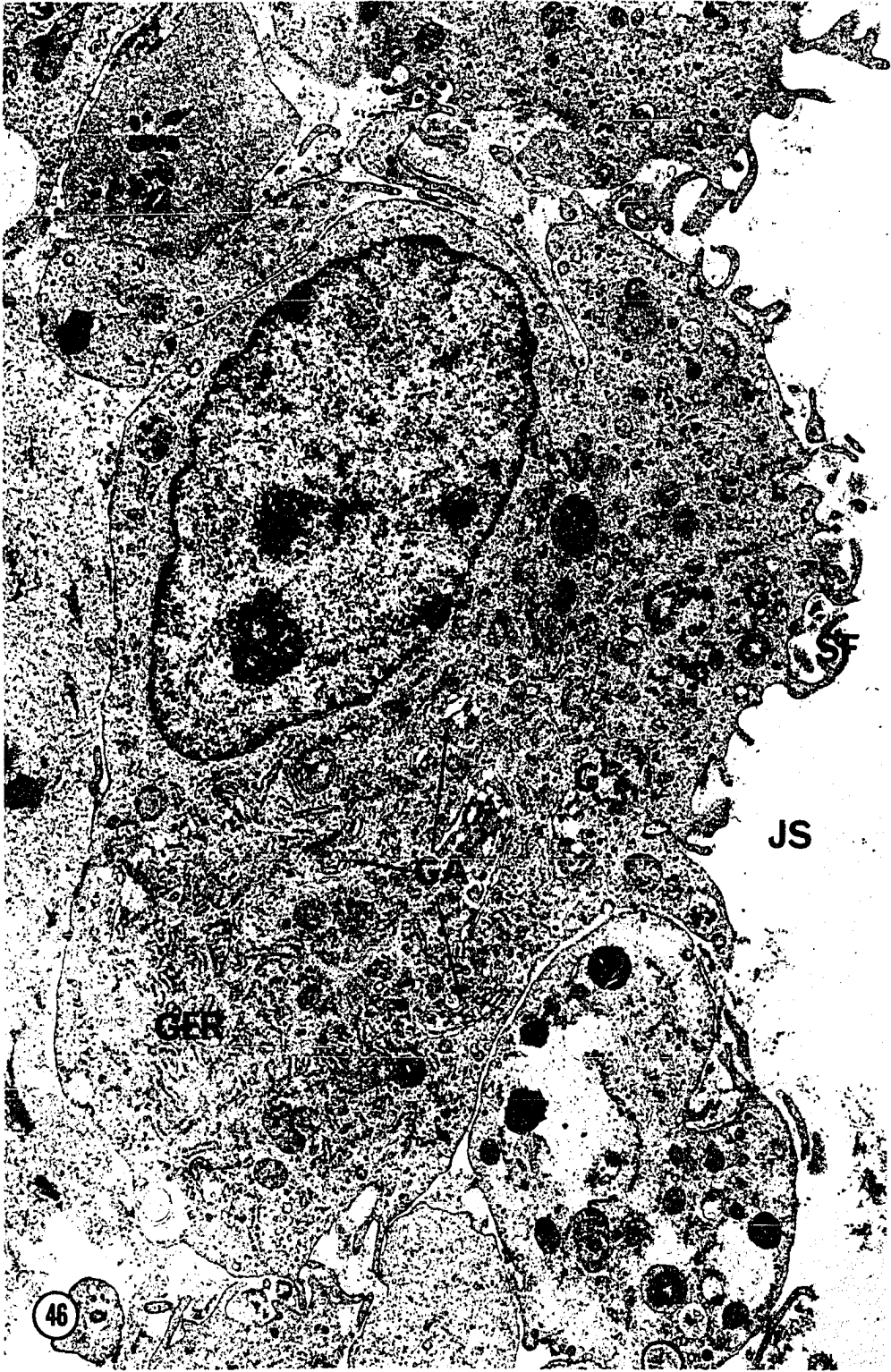


Figure 47. Polarized lining cell after 21 days of infection. Note the similarity to a type A lining cell at the apex and similarity to a type B lining cell at the base. X 14,000

- G - granules
- GER - granular endoplasmic reticulum
- JS - joint space
- LV - large vesicles
- M - mitochondria
- Nuc - nucleus
- R - free ribosomes
- SV - small vesicles



Figure 48. Two polarized lining cells 21 days postinoculation. Each cell contains granules (G) in the apex and granular endoplasmic reticulum (GER) at or near the base. Note the dilated endoplasmic reticulum and greater electron density of cell at right and structure resembling a residual body (RB) in cell at left. Uranyl acetate and lead citrate. X 8,700

G - granules
GA - Golgi apparatus
GER - granular endoplasmic reticulum
JS - joint space
M - mitochondria
RB - residual body

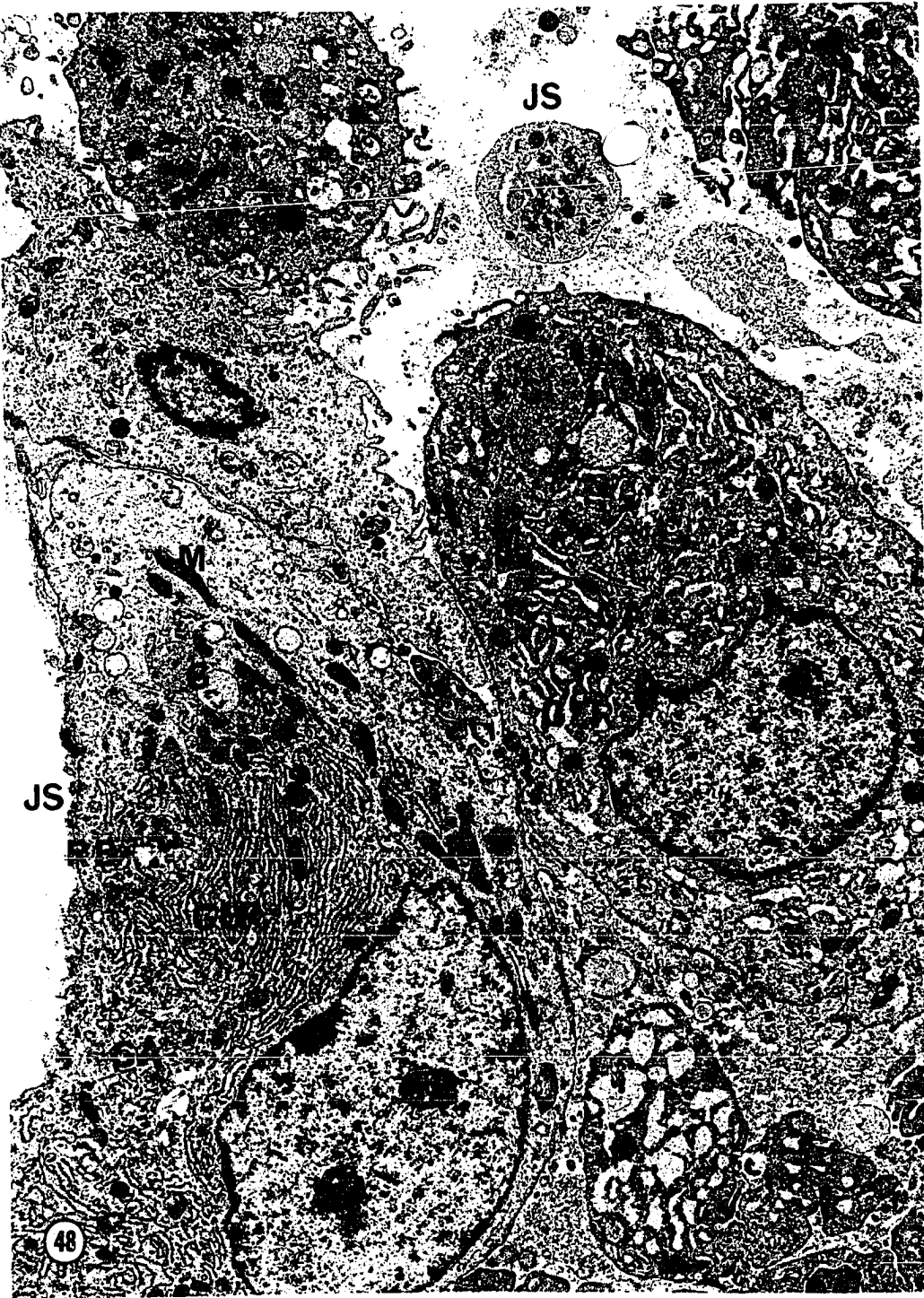


Figure 49. Type A synovial cells (A) containing cytoplasmic granules (G) up to 1.5 microns in diameter, after 21 days of infection. The edge of a type B cell (B) is seen at the upper left. Intercellular vesicles (PRV) are interpreted as dilated endoplasmic reticulum from ruptured plasmacytes. Uranyl acetate and lead citrate. X 8,700

A - type A synovial lining cells
B - type B synovial lining cells
G - granules
JS - joint space
PRV - plasmacyte-reticulum vesicles



Large cytoplasmic protrusions or blebs were attached to a few polarized and type A cells and appeared in cross section in the matrix surrounding the lining cells (Figure 50). They contained a fine granular substance similar to the intercellular matrix of the synovial lining. Membranes of some cytoplasmic protrusions were ruptured with an apparent release of intracytoplasmic contents.

Neutrophils were the predominant inflammatory cells in the synovium and synovia of infected joints (Figures 42 and 43). They were in greatest concentration at 48-96 hours postinoculation, often interspersed with fibrin; most were intact but some were fragmented with release of granules. Eosinophils and mast cells were found occasionally.

Lymphoid cells were found in the synovium of infected joints with an increasing frequency as the lesion progressed. After 10 days of infection, they were seen in large numbers mixed with numerous cells of the plasmacytic series (Figure 51). As classified by Leduc et al. (1968), these cells were lymphocytes, hemocytoblasts, plasmablasts, immature plasmacytes, and mature plasmacytes. After 21 days, many plasmacytes were ruptured with cytoplasmic constituents in the intercellular space.

Many macrophages were found in the synovium, subsynovium, synovia, and exudate of infected joints (Figures 52, 53 and 54). They were recognized by the presence of large phagocytic vacuoles (phagosomes) containing cellular debris and fibrin.

Figure 50. Synovium, 21 days postinoculation, with cytoplasmic protrusions (CP) of lining cells (LC) extending into the (a) joint space (JS) and (b) intercellular matrix (IM). (c) Cross sections of the protrusions with light granular contents similar to the extracellular matrix. Note the ruptured plasma membrane of the cytoplasmic protrusions in micrographs a. and b. Uranyl acetate and lead citrate. a. X 5,000; b. X 6,200; c. X 14,000

CP - cytoplasmic protrusions of lining cells
IM - intercellular matrix
JS - joint space
LC - synovial lining cells
PRV - plasmacyte-reticulum vesicles
RBC - erythrocytes

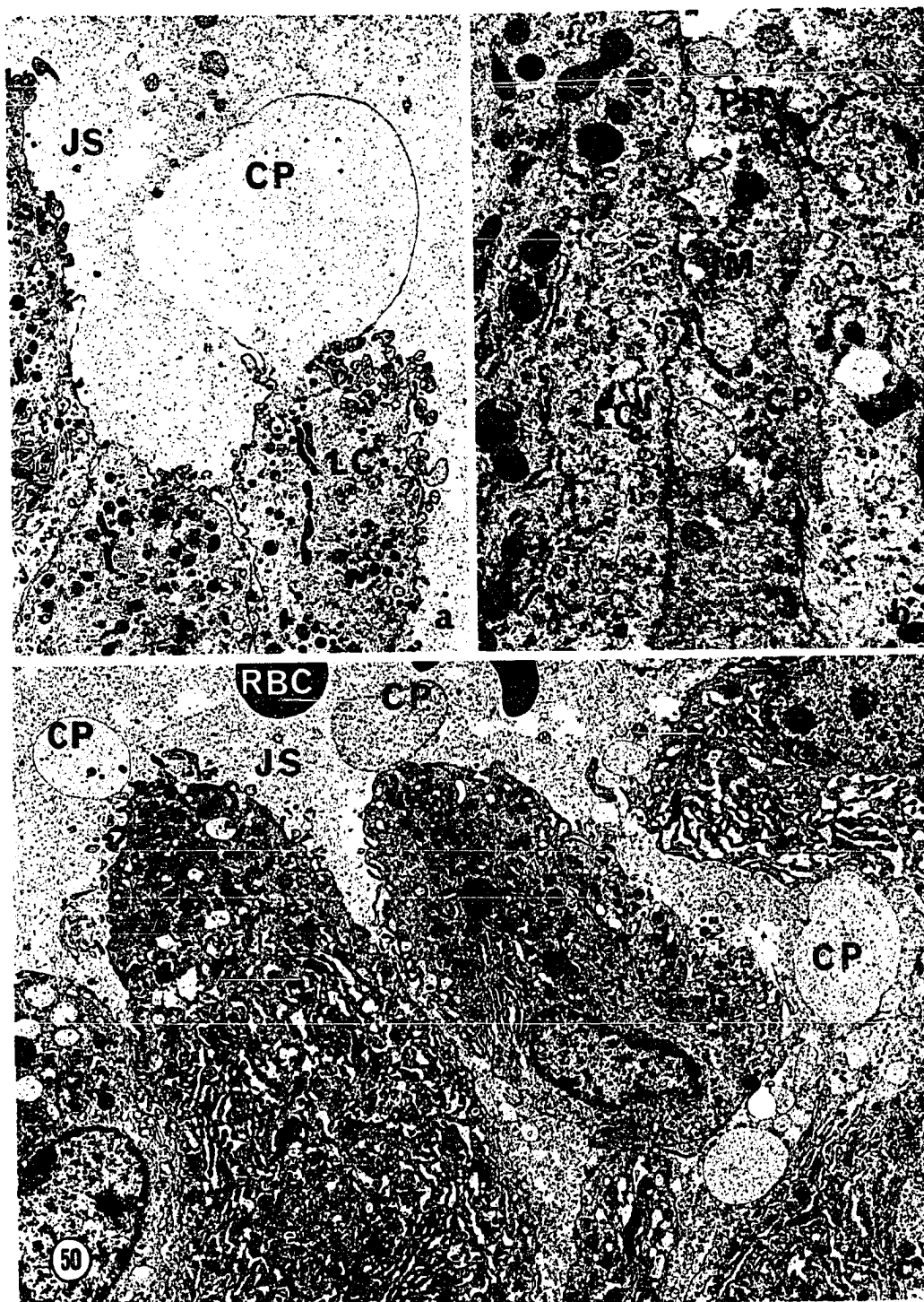


Figure 51. Chronic synovitis from lambs affected with OCP. (a) Twelve days and (b) 10 days post-inoculation, cells of the lymphoid and plasmacytic series can be identified. (c) Twenty-one days postinoculation, degenerate plasmacytes consisting of shrunken nuclei (Nuc) surrounded by vesicles (PRV) of granular endoplasmic reticulum. Uranyl acetate and lead citrate. X 4,400

Co - collagen
HB - hemocytoblast
IPC - immature plasmacyte
L - lymphoid cell
MPC - mature plasmacyte with Russell
bodies
Nuc - nucleus
PB - plasmablast
PRV - plasmacyte-reticulum vesicles

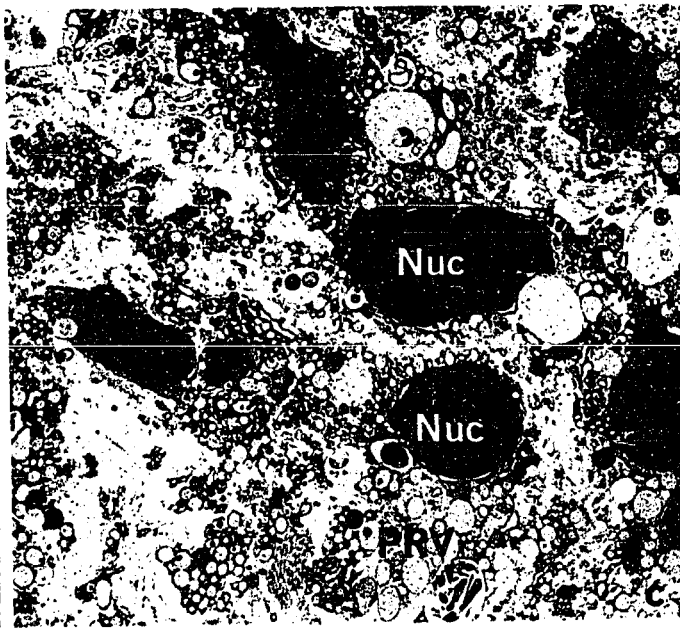
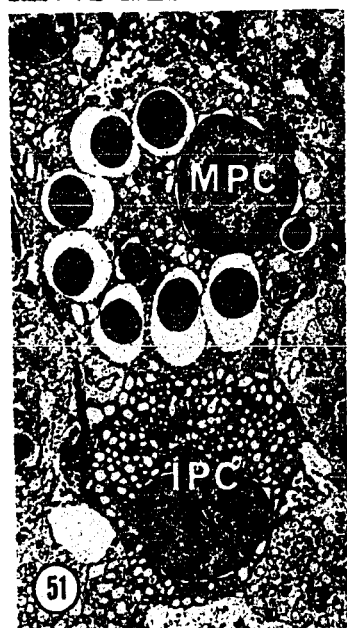
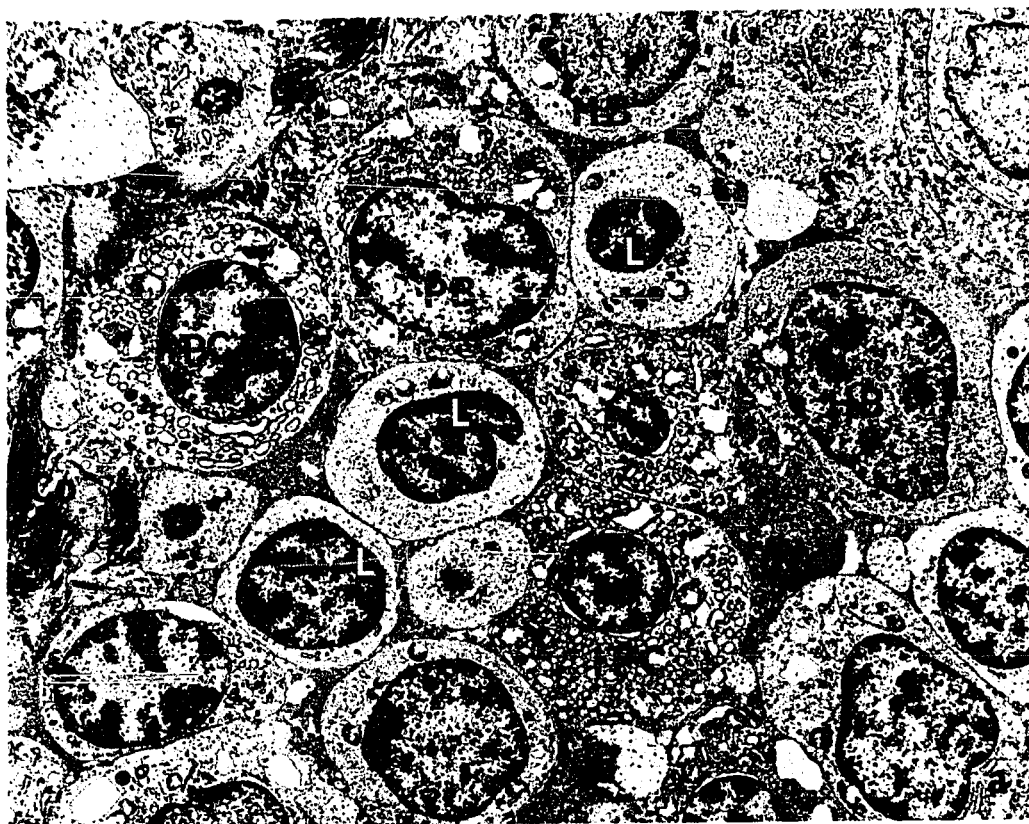


Figure 52. Phagocytic cell from the synovial fluid of an intravenously infected lamb. This cell has many characteristics of the type A lining cell, including large cytoplasmic granules (G), small vesicles (SV), and surface folds (SF) which appear to be forming pinocytotic vesicles. The large phagocytic vacuole (PV) contains apparent neutrophil granules. Uranyl acetate and lead citrate. X 14,000

G - granules
PV - phagocytic vacuole
SF - surface folds
SV - small vesicles

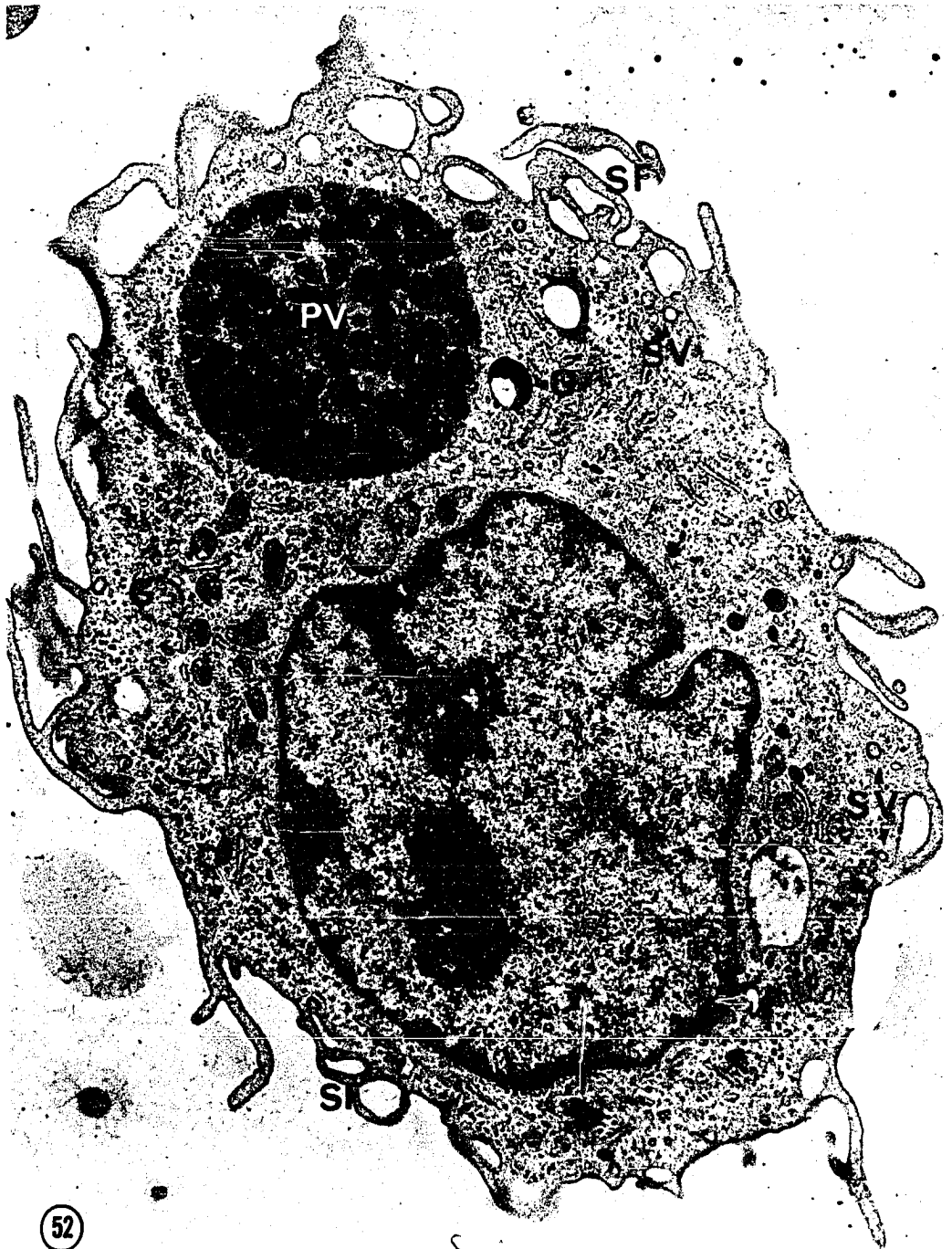
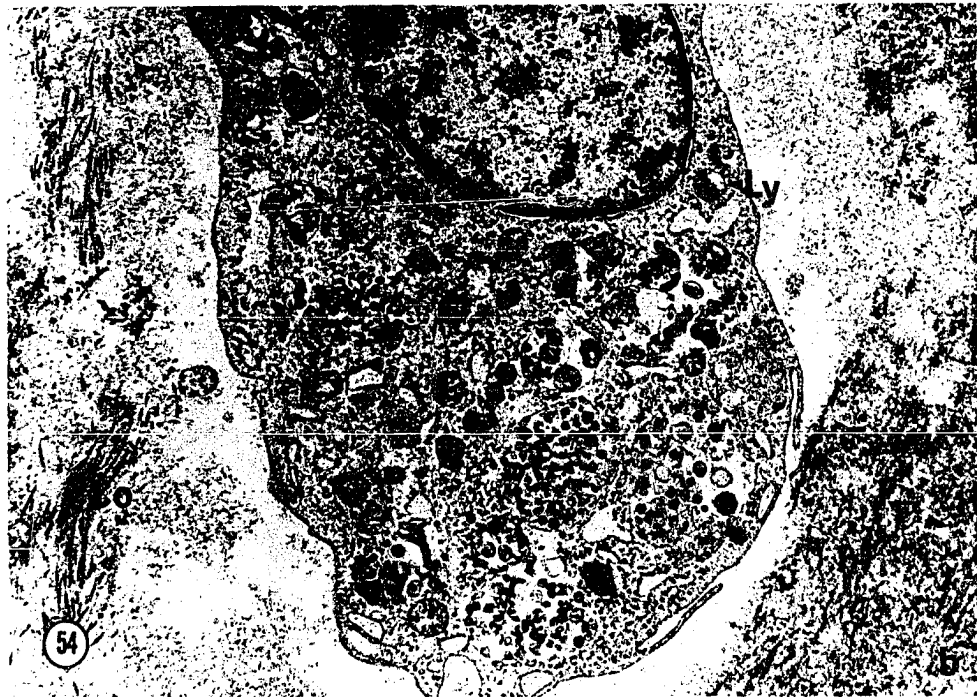
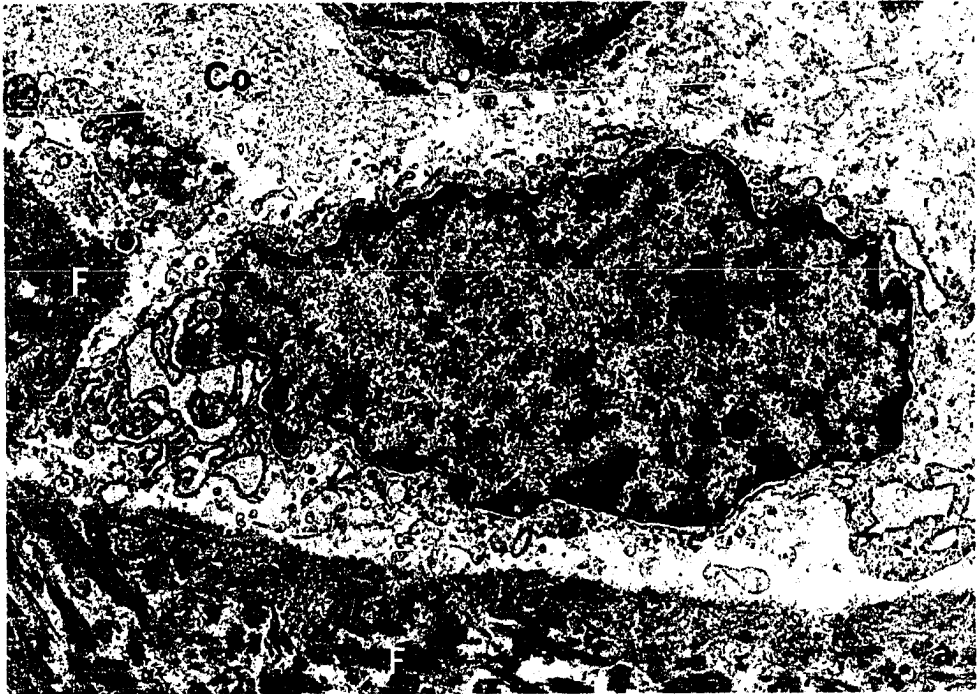


Figure 53. Phagocytic cell from the synovial fluid of a lamb 12 days after intraarticular injection of the OCP agent. This cell contains several large phagocytic vacuoles (PV) and may represent a monocytic type cell. Uranyl acetate and lead citrate. X 18,000



Figure 54. Cells, (a) fibroblast and (b) macrophage, from an area of organizing fibrinous exudate in the synovium 21 days postinoculation. Multiple phagocytic vacuoles (PV) which contain cellular debris and fibrin, and apparent lysosomes (Ly) are present in the macrophage. Collagen (Co) fibers surrounding the cells are presumably being produced by fibroblasts. Uranyl acetate and lead citrate. a. X 10,000; b. X 8,700

Co - collagen
F - fibrin
GA - Golgi apparatus
Ly - lysosomes
PV - phagocytic vacuoles



Similar cells, without phagosomes, were also widely distributed in the infected tissues (Figure 43). Whether these are synovial cells or monocytes is uncertain; however, well differentiated type A or B synovial cells were not found with obvious phagosomes.

Fibroplasia was apparent in the synovium of lambs after 72 hours of infection. Many fibroblasts surrounded by collagenic fibers were interspersed through the fibrinous exudate in association with macrophages containing phagocytized fibrin and cellular debris (Figure 54). Similar areas as well as large areas of highly vascularized fibrous tissue were found in the synovium of lambs killed later than 96 hours post-inoculation.

Chlamydial organisms were encountered in 4 of the 29 joints that had been infected by the intraarticular route. These were found in lambs killed 14, 24, and 48 hours and 12 days after inoculation. None were found in lambs with the naturally occurring disease or in those given the agent intravenously. Organisms found were either in extracellular debris or within the cytoplasm of phagocytic cells, either neutrophils or macrophages (Figures 55 and 56). Most were of the small particle type and located within intracytoplasmic vacuoles or phagosomes; often only remnants remained. Large particles were found less frequently and their identity was less definite. Numerous other structures were present in the macrophages and synovial lining cells which at low magni-

fication were indistinguishable from chlamydiae; however, at higher magnification, their morphologic detail was not characteristic of chlamydial particles but were single-membrane-bound granules.

Figure 55. Chlamydial organisms phagocytized by neutrophils in the synovium of a lamb killed 14 hours postinoculation. Small chlamydial particles (SP) are present (a) within a phagocytic vacuole and (b) free in the cytoplasm surrounded by what may be lysosomal material; possible organisms (arrows) can be seen in a phagocytic vacuole. These organisms apparently were being destroyed by lysosomal enzymes. Uranyl acetate and lead citrate.
X 41,000

F - fibrin
Ly - lysosomes
Nuc - nucleus
SP - small chlamydial particles
Arrows - possible degenerate chlamydiae

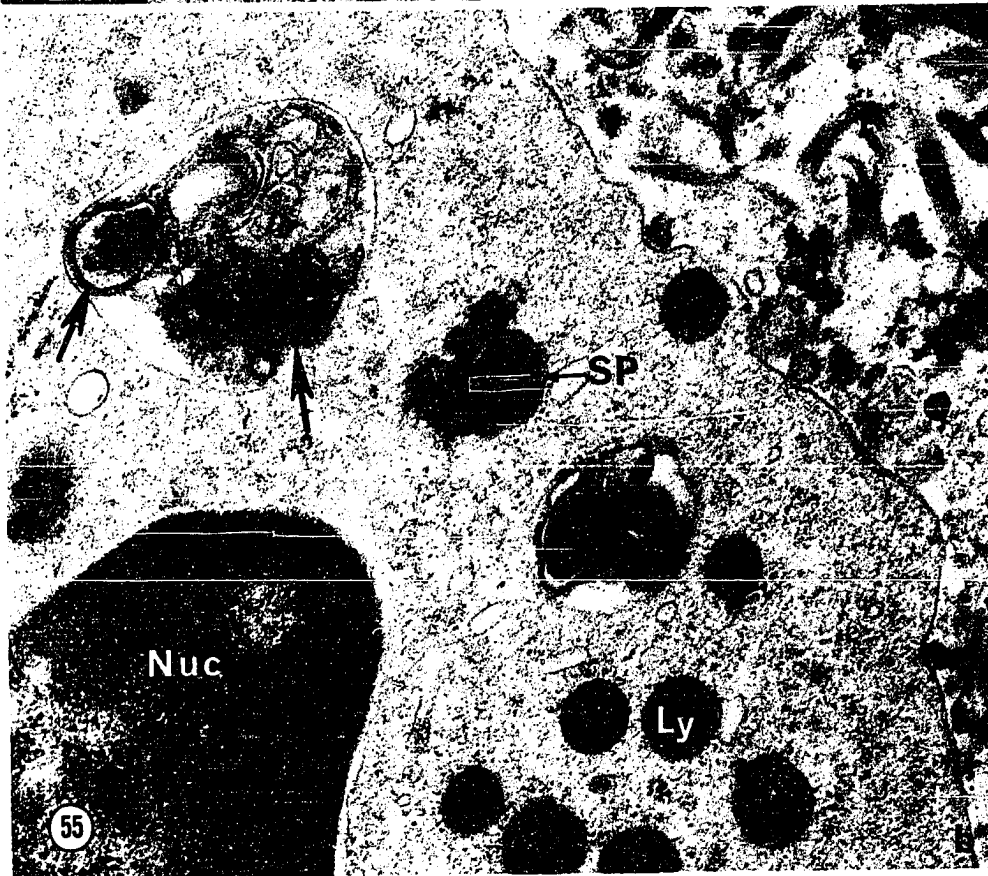
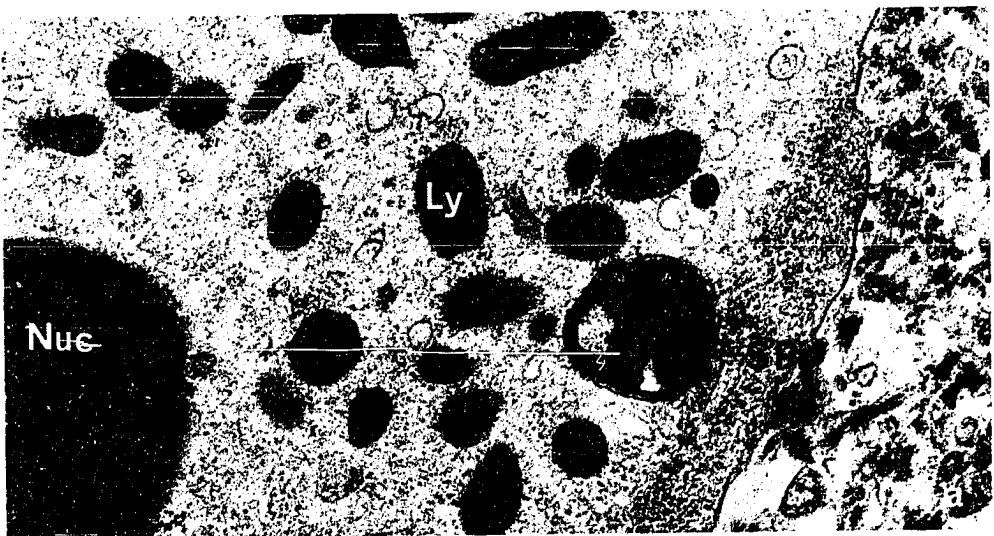
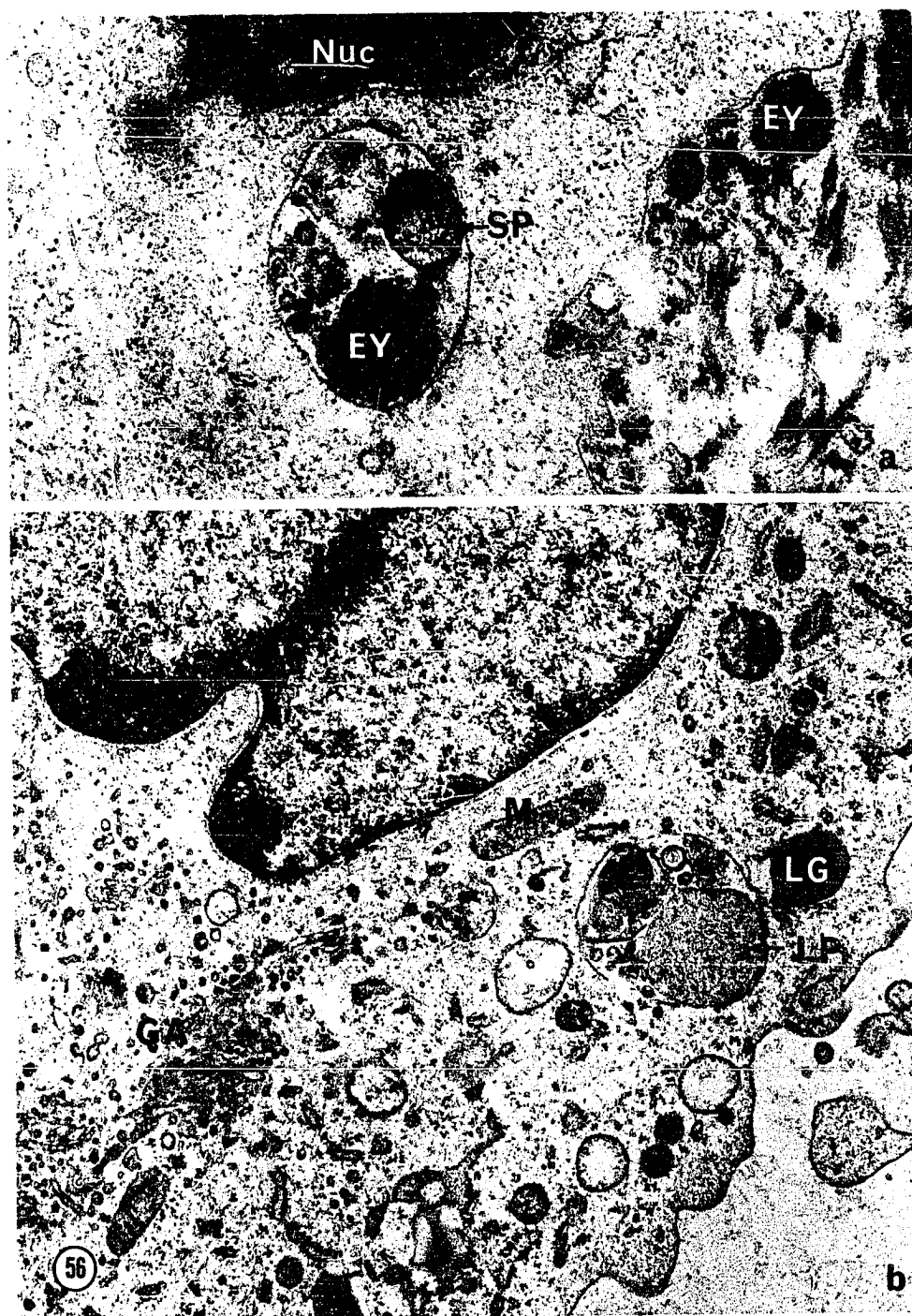


Figure 56. Chlamydial particles in phagocytic vacuoles of macrophages from (a) the synovial membrane of a lamb killed 14 hours after inoculation and (b) the synovial fluid of a lamb killed 48 hours after inoculation. The electron-dense material in the vacuole and extracellular space in micrograph a. is interpreted as egg yolk (EY). Uranyl acetate and lead citrate. a. X 41,000; b. X 24,000

EY - egg yolk
F - fibrin
GA - Golgi apparatus
LG - lipid globule
LP - large chlamydial particle
M - mitochondria
Nuc - nucleus
SP - small chlamydial particle



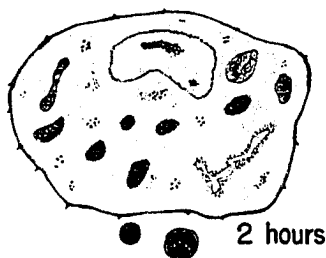
DISCUSSION

Morphology and replication of the OCP agent were studied in McCoy cell cultures by electron microscopy and will be considered together because of their intimate interrelationship. The replicative cycle could not be followed in synovial tissues since too few organisms were found. In cell cultures, growth of the organism was similar to other chlamydiae examined since 1964 (Armstrong and Reed, 1964; Higashi, 1964, 1965; Mitsui et al., 1967). Replication occurred in successive stages without loss of morphologic identity. Small particles were phagocytized by cells and developed within the cytoplasm into large particles which divided by binary fission; the daughter organisms either repeated the division or condensed to form new small particles which were released by rupture of the cell membrane. A schematic representation of this cycle is presented in Figure 57.

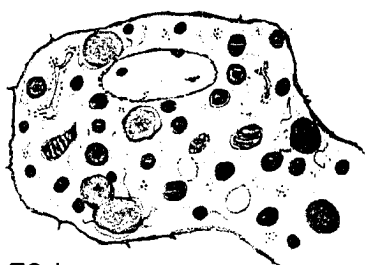
Phagocytosis occurred by cytoplasmic processes or pseudopodia of the cells surrounding and engulfing small particles of the organisms. Shortly after small particles were phagocytized by cells, or possibly upon contact with the cytoplasmic membrane, they began to enlarge and developed into large granular particles. Enlargement was associated with dispersal of the dense chromatin nucleoid, rearrangement of the multilaminated wall, and tremendous growth.

Fluorescent antibody staining during this phase of devel-

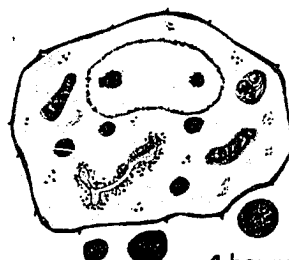
Figure 57. Scheme of replication of the OCP agent in McCoy cells. Two and 4 hours postinoculation small organisms are located extracellularly and by 8 hours one is being engulfed by a cell. Phagocytosis continues at 24 hours plus large particles of the organism are present within the cell cytoplasm. By 48 hours postinoculation the cycle is complete with newly formed small particles interspersed among large particles which continue to divide by fission. Organisms are released by rupture of the cell 72 hours after inoculation



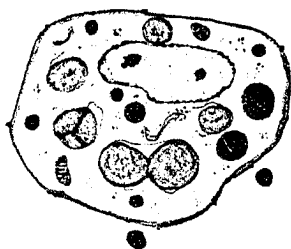
2 hours



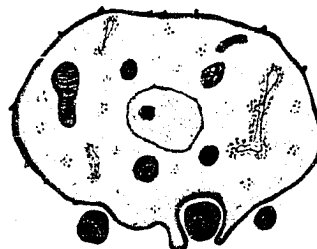
72 hours



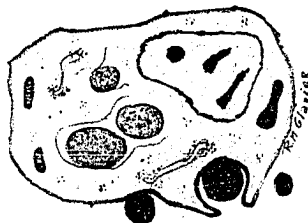
4 hours



48 hours



8 hours



24 hours

opment was considered negative because of inability to differentiate minute fluorescent foci from those present in control cells. Probably inclusions of the OCP agent did stain but were too small to be recognized since only single particles were found by electron microscopy. Thus, failure of detectable staining cannot be interpreted to represent loss of antigenicity as the result of an eclipse as has been reported for other chlamydiae (Pollard and Tanami, 1962). Antigens of the 6BC strain of the psittacosis agent have been shown to be present throughout the developmental cycle (Buckley et al., 1955).

Dispersal of the dense chromatin nucleoid of the small particle was associated with appearance of strands of deoxyribonucleic acid in the granular matrix of the large particle. Ribosomes, present in all organisms, were typical of single free ribosomes in other cells; these have been isolated from the large particle form by Tamura (1967) and resemble ribosomes of other bacteria in buoyant density. The multilaminated wall of the small particle gradually changed into 2 trilaminar membranes surrounding the large particles. The latter was also surrounded by the membrane of the phagocytic vacuole which was ruptured soon after division began. Membranes of large particles closely resembled the plasma membrane and cell wall of other gram-negative bacteria as reported by von Iterson (1965). Jenkin (1960) found the walls of the meningopneumonitis agent chemically similar to those

of rickettsiae and gram-negative bacteria.

Division of the large particles was principally by simple binary fission. Internal membranes found within some large particles (Figure 13) may represent fission into 3 or more daughter cells or faulty attempts at binary fission; however, these forms were numerically insignificant when compared to dumbbell shapes representing binary fission. Organisms with internal membranes of this type but with the contents shrunken during processing for examination, probably are similar to those interpreted by others (Gaylord, 1954; Bernkopf et al., 1962; Higashi et al., 1962) to represent endosporulation. Endospores were not found in this study nor were particles found which were believed to represent unequal fission as proposed by Tajima et al. (1957) and by Litwin et al. (1961) for several other chlamydiae. Unequal dumbbell profiles of the OCP agent were interpreted as resulting from the plane of the section and not from unequal fission.

After division, daughter particles either continued to divide, apparently after enlargement, or condensed to form mature small particles. This maturation process was accomplished by condensation of the chromatin, which at first was located centrally, forming the intermediate particle, and finally located at the periphery of the particle, and by rearrangement or contraction of the limiting membranes into an apparently rigid multilaminated wall. A similar process of maturation has been suggested for several other chlamydial

organisms; however, Higashi (1964) reported that the trachoma agent matured by forming a new membrane around a shrunken central core with disappearance of the former external membrane. Microtubules found in a few large particles are of unknown significance but may be important in the maturation process. Fawcett (1966) stated that microtubules found in the cytoplasm of many cells are considered to be resilient cytoskeletal elements that aid in maintaining cell shape by imparting a certain stiffness to the cell. He also suggested that microtubules probably have contractile properties and may be involved in the internal movement of cells and alterations of cell shape. If this assumption is correct, contraction of microtubules in large chlamydial particles may be a significant means of size reduction during formation of the mature small particle.

The mature small particles were generally composed of an electron-opaque nucleoid in an eccentric location and a fine granular matrix containing ribosomes. Small organisms without a visible nucleoid are probably attributable to the plane of the section. The multilaminated wall of the small particle appeared to be composed of 2 trilaminar membranes tightly apposed to the contents of the organism. This was best seen in a few extracellular organisms where the membranes had separated (Figure 7) and is in agreement with the speculation of Anderson et al. (1965) that 2 membranes invest the small particle of the lymphogranuloma venereum agent.

Division and maturation of intracellular particles continued until the plasma membrane of the cell ruptured and the organisms were released. As observed with the electron microscope, this cycle required 48 hours for formation of mature small particles and 72 hours for release of the organisms. The possibility of sampling errors and the use of nonpurified inoculum resulting in possible unsynchronized infection may have influenced the apparent replication time.

The results of acridine orange staining of chlamydial-infected cell cultures were interpreted by Pollard and Starr (1962) and Pollard and Tanami (1962) to indicate a true viral replication. They reported a change in the staining characteristics of the agents of psittacosis and trachoma at various stages of growth. In contrast, no change of color was detected during sequential development of the OCP agent; inclusions fluoresced bright orange before treatment with ribonuclease and green after treatment, indicating, according to Mayor (1962), the presence of ribonucleic acid and deoxyribonucleic acid in all stages of development. This is in general agreement with the observations of Furness and Csonka (1963) on the development of the agent of lymphogranuloma venereum. However, they found a gradual change in color of fluorescence from red, which was correlated with the presence of large particles, to yellow-green with the appearance of small particles. Green fluorescence, indicating pure deoxyribonucleic acid, was found only after treatment with ribo-

nuclease. Chemical analyses have shown that small particles of the meningopneumonitis agent are approximately equal in content to ribonucleic acid and deoxyribonucleic acid (Tamura and Higashi, 1967); whereas, large particles contain approximately 3 times more ribonucleic acid than deoxyribonucleic acid (Tamura et al., 1967). It is likely that orange fluorescence in late infection with the OCP agent was the result of many large particles in the cellular inclusions; even though the small particles predominated.

Enzyme histochemical procedures were uninformative other than indicating infection of McCoy cells with the OCP agent did not alter enzymatic activity to a degree that could be detected by the procedures used. Any difference in enzymatic activity of synovial cells in infected and noninfected joints was overshadowed by the intense activity in inflammatory cells. Similarly, oil red O staining lipid was not altered by infection.

Dissemination of the OCP agent to other joints following intraarticular injection was not detected as reported by Storz et al. (1965). Virulence of the respective organisms apparently was not a factor in this difference since their pathogenicity for 8 animal species was compared and found to be identical.¹ However, failure of the agent to spread to other

¹Page, L. A., National Animal Disease Laboratory, Ames, Iowa. Comparative pathogenicity of two isolates of the OCP agent. Personal communication. 1970.

joints may have been the result of a resistance in the lambs which was not measured by the complement fixation test. Such a resistance may explain the small number of organisms found in synovial tissues. Apparently many organisms were destroyed by phagocytic cells, as seen with the electron microscope, soon after injection or after release from the few cells which were infected. It is questionable if sufficient individual cells were infected to cause the rapid and extensive articular damage which resulted from intraarticular injection of the OCP agent. Rather, it is likely that the organism contains an inflammation-producing toxin as has been demonstrated in the cell wall of other chlamydiae (Ross and Jenkin, 1962; Christoffersen and Manire, 1969).

Sequential development of articular lesions can be divided into 3 stages, serous inflammation from 2-14 hours postinoculation, fibrinopurulent inflammation from 24-96 hours postinoculation, and resolution 6-24 days postinoculation. Serous inflammation was characterized by excess synovial fluid, edema of the synovium, and emigration of neutrophils. Destruction of synovial lining cells and fibrinopurulent exudation rapidly followed. Intact synovial lining cells were hyperplastic and hypertrophic with increased basophilic staining of the cytoplasm of many cells. Resolution of the lesion, although not complete, was characterized by extensive fibrosis, formation of lymphoid follicles, plasmacytosis, and recovering of the denuded synovial surface with

lining cells. Others (Shupe and Storz, 1964; Storz et al., 1965) considered accumulations of mononuclear cells as a granulomatous reaction. Foci of mononuclear cells in lambs of this study were not those of a granulomatous response but were lymphocytes and developmental forms of plasmacytes as outlined by Leduc et al. (1968). Such cells apparently function in local production of antibody. No changes were found in articular cartilages as reported by Shupe and Storz (1964).

It was necessary to first study the ultrastructure of normal synovium in order to evaluate changes caused by infection. Normal synovium was found to vary from region to region within the joint; one variety was believed to correspond to the fibrous membrane and the other to the areolar membrane of light microscopy. Lining cells of the fibrous membrane were indistinguishable from underlying fibroblasts; whereas, the areolar membrane was structurally similar to the synovium of man as described by Barland et al. (1962). There were no specialized areas of intercellular connections between the lining cells. Collagenous fibers were frequently located in the intercellular space of the lining cells but were found not to extend to the synovial surface as reported by some authors for other species (Coulter, 1962; Roberts et al., 1969).

Barland et al. (1962) first intimated a functional relationship between type A and B lining cells and considered

the possibility of a single cell type in different functional states in the synovial lining of man. This view was also held by Luckenbill and Cohen (1967) who considered only a single cell type to be present in the synovium of chickens. The synovial lining of lambs was found to be made up of 2 distinct cell types with a few cells characteristic of neither but a direct morphologic link between the two could not be established in normal synovium. However, in the synovium of lambs with OCP there was a morphologic transition between A and B cells during what appeared to be a maturation process. Following hypertrophy of the type B cells in the early stages of infection, they developed into polarized cells with large granules characteristic of type A cells in the apex and granular endoplasmic reticulum in the base similar to type B cells. The mature type A cell appeared to arise by formation of additional large granules accompanied by a gradual dissolution of the granular endoplasmic reticulum. In normal synovium the polarized cell apparently does not form; instead, the transition appears to be direct from B to A type cells by a less distinctive process of granule production and loss of endoplasmic reticulum.

The large granules are believed to be a secretory product possibly formed by coalescence of Golgi vesicles. Chemical composition of the granules is not known; however, the granular endoplasmic reticulum of the type B cell indicates protein production and the extensive Golgi apparatuses of both types

of cells may be active in the production of hyaluronate. Involvement of the Golgi apparatus in the synthesis of complex carbohydrates has been shown by the radioautographic studies of Peterson and Leblond (1964). Thus granules of the lining cells may consist of hyaluronateprotein. In addition to ultrastructural evidence, this suggestion is supported by the demonstration by immunofluorescence of hyaluronateprotein in lining cells (Blau et al., 1965) and by the finding that several proteins, originally believed to be of plasma origin, are produced by synovial lining cells (Williamson et al., 1966). Hypertrophy, which included an increased amount of granular endoplasmic reticulum, of type B lining cells during infection with the OCP agent indicates increased production of protein which may account for the increased size of the secretory granules as compared to those of non-infected synovium. A similar hypertrophy may also explain the increased protein of the hyaluronateprotein complex found by Hamerman and Sandson (1963) in some pathologic joint processes of man.

Several possible mechanisms could be involved in the release of secretory products from the lining cells. One possibility is an apocrine secretory process akin to that demonstrated by Trier (1968) in intestinal crypt epithelium. Cytoplasmic protrusions of the lining cells project into the joint cavity and intercellular space, rupture, and release their content (see Figure 50). The possibility exists that

such cytoplasmic protrusions do not represent active secretion but rather are indicative of cellular degeneration. In either event, the result would be the same, release of cytoplasmic content. A second possible mechanism of releasing the secretory granules is sloughing of the mature type A cells from their superficial location into the joint cavity and their subsequent rupture. Such a mechanism does not explain the presence of hyaluronateprotein in the intercellular areas of the synovial lining as has been shown by Blau et al. (1965). Another possibility is that secretion occurs by transport of a product via small vesicles to the plasma membrane of the cell with release of the contents by a process of reverse pinocytosis.

However, most if not all of the small vesicles at the cell membrane are believed to be micropinocytotic vesicles. Pinocytosis is probably necessary to explain the uptake of colloidal carbon and iron dextran by lining cells as has been demonstrated in other species (Ball et al., 1964; Luckenbill and Cohen, 1967). Also, the presence of lysosomes in synovial lining cells of man, pigs (Hamerman et al., 1961; Roberts et al., 1969), and lambs, as indicated by acid phosphatase activity, signify these cells are active in the uptake and hydrolysis of some extracellular substance. Whether lining cells actively phagocytize particulate matter, such as cellular fragments, is questionable. Cells in the synovial fluid similar to type A lining cells were found to contain large phagocytic

vacuoles; however, such vacuoles were not found in similar cells in the intact synovium. Phagocytic cells in disrupted areas of the synovium were believed to be more characteristic of monocytes than of lining cells; however, the morphologic differentiation of such cells is difficult.

Detection of the OCP agent in infected cell cultures was easiest with the fluorescent antibody technique; fluorescent inclusions were found in all infected cultures after 24 hours of incubation. Minute fluorescent foci, some of which probably were organisms, in cells incubated for less time could not be differentiated satisfactorily from similar foci in control cells. Giemsa staining was much less acceptable because of failure to stain many inclusions, especially small ones of early infection, and less distinct staining of others. These conclusions agree with those of Buckley et al. (1955) and Nichols et al. (1963) on the agents of psittacosis and trachoma respectively. Also, the nonspecificity of Giemsa stain is disadvantageous because of the difficulty in distinguishing chlamydiae from cytoplasmic and nuclear debris (Gikes et al., 1958). Acridine orange, although lacking the specificity and brilliance of staining, paralleled fluorescent antibody for detecting quantity of inclusions. Enzyme antibody staining was satisfactory only in 96-hour and 240-hour cultures. Excess stain precipitate occurred in all slides, making difficult the detection of smaller inclusions. However, this technique, with refinements, appears promising

for supplementing fluorescent antibody methods. It retains specificity for antigens, eliminates the need for an ultra-violet light source, results in permanent preparations, and has the potential for use in electron microscopy (Nakane and Pierce, 1967). Because of their distinctive ultrastructure, particles of the OCP agent were readily recognized in infected McCoy cell cultures with the electron microscope.

Few organisms were found in the joints of lambs either by light or electron microscopic techniques; even though the agent was injected intraarticularly in an attempt to infect a large percentage of cells. In contrast, Norton and Storz (1967) reported recognizing the OCP agent in infected joints with relative ease. However, illustrations of their intercellular particles do not have the characteristic ultrastructure interpreted as the OCP agent in this study or of other chlamydiae. Structures such as they illustrate were found in this study and were thought to represent necrosis or phagocytosis of cellular fragments. Failure to find many organisms with the electron microscope was believed not to be a sampling error since only a few organisms were found with fluorescent antibody staining. Results of staining with fluorescent antibody were confusing because of the large number of eosinophils in the tissues which fluoresced similar to chlamydial inclusions in McCoy cells. However, a few scattered cells with cytoplasmic fluorescence, found not to be eosinophils by restaining the sections with Giemsa,

were believed to contain the OCP agent. Giemsa stained organisms, if present, were overshadowed by intracytoplasmic cellular debris in cells either in smears or sections. Similarly, fluorescence of cellular ribonucleic acid in the inflammatory lesion obliterated any possible staining of organisms with acridine orange. Diffuse staining of synovial tissues with peroxidase labeled antibody was apparently caused by endogenous peroxidase or pseudoperoxidase.

Observations made during this study indicate that the OCP agent is a bacterium as suggested by Moulder (1966) for all chlamydiae. It was shown that the OCP agent contains both ribonucleic acid and deoxyribonucleic acid, divides by binary fission, and maintains a morphologic identity throughout the developmental cycle; any one of which is sufficient to separate this agent from viruses. Classification of members of the genus Chlamydia into 2 species based on morphologic and physiologic characteristics has been proposed by Page (1968). One criterion for classification was the presence or absence of glycogen associated with intracellular organisms. The faint periodic acid-Schiff reaction, failure of the reaction to be altered by diastase, and electron microscopic observations indicate the absence of glycogen in cells parasitized by the OCP agent. Another standard used to divide the genus was the type of intracellular colony. Intracellular organisms were reportedly found either in compact, membrane-limited microcolonies or diffusely distributed throughout

the cytoplasm. Electron microscopy revealed that the agent of OCP conformed to the latter division, the intracytoplasmic colonies being surrounded by membrane of the phagocytic vacuole only in early infections, later it was ruptured and organisms were scattered throughout the cytoplasm.

The noncytopathic virus contaminant found in McCoy cells during the course of this study structurally resembled type C murine leukemia virus as classified by Bernhard (1960). Virions of this type have been found in the tissues of leukemic mice, disease free conventional mice, and germfree mice (Bernhard, 1960; de Harven, 1964; Kajima and Pollard, 1965). Their significance in nonleukemic mice is undetermined but they may represent latent forms of leukemia virus. This observation of type C virions in McCoy cells, supplements previous reports (Dmochowski et al., 1967; Kajima et al., 1967) and tends to corroborate general contamination of McCoy cells. Dmochowski et al. (1967) reported that McCoy cells are not human synovial cells as originally characterized; instead, immunologic and karyotyping techniques indicate they are of murine origin. This supports the ultrastructural evidence that the virus is a murine leukemia or closely related virus. However, additional information on its biologic activity is needed to accurately determine its nature.

SUMMARY AND CONCLUSIONS

McCoy cell cultures and articular tissues of 24 lambs infected with the agent of ovine chlamydial polyarthrititis were examined by light and electron microscopy in an attempt to determine the structure and mode of replication of the agent, to study the pathologic alterations, and to evaluate methods of detecting the organism. Synovial tissues were examined from 18 lambs exposed intraarticularly, 2 exposed intravenously and 4 with spontaneous disease.

As seen in thin sections of infected cell cultures, the organism exists either as small (250-450 nm) electron-dense particles with an eccentric nucleoid, ribosomes, and a multilaminated cell wall or as large (600-1500 nm) granular particles containing ribosomes, strands of deoxyribonucleic acid, and enveloped by 2 unit membranes. Particles, intermediate in size and structure to the 2 major types, were also present. Replication, which occurred throughout the cytoplasm, was initiated by phagocytosis of small chlamydial particles. Upon entering a cell, they developed into large particles which divided by binary fission. Daughter particles either repeated the division or condensed to form new small particles. The replicative process was terminated by rupture of the plasma membrane of the cell. This cycle was complete with formation of new particles by 48 hours postinoculation. Failure to find sufficient organisms precluded a description

of the organism in ovine synovial tissues.

Pathologic changes in the joints of lambs following intraarticular injection of the OCP agent occurred in 3 stages; an initial serous synovitis and tenosynovitis 2-14 hours postinoculation, followed by fibrinopurulent inflammation 24-96 hours postinoculation, and finally early changes of resolution 6-24 days postinoculation. Serous inflammation was characterized by hyperemia and edema of the synovial tissues, emigration of neutrophils into the joint, and excessive synovial fluid containing a few flakes of fibrin. By 24 hours after inoculation areas of necrotic synovium were covered by thick layers of fibrinopurulent exudate and the synovial fluid contained numerous neutrophils and large flakes of fibrin. Areas of intact synovium had hyperplastic and hypertrophic lining cells. Early changes of resolution of the articular lesion were present in the lambs affected longest. These included organization of the fibrinopurulent exudate, recovering of the areas of denuded synovial surfaces with lining cells, and infiltration and proliferation of lymphocytes and plasmacytes. Changes in the joints of lambs infected intravenously were similar to the early changes in intraarticularly infected lambs; whereas, joints of naturally infected lambs were similar to those of lambs infected intraarticularly for the longest period of time.

Ultrastructure of normal synovial membranes was studied for comparison with infected membranes. Some areas of syno-

vium corresponded to the fibrous membrane of light microscopy where the lining cells rested on dense collagenous tissue and were indistinguishable from underlying fibroblasts. Other areas, corresponding to the areolar membrane of light microscopy, were characterized by lining cells similar to Barland's type A and B cells of man overlying loose fibrous tissue containing many vessels. Type A cells differed from type B cells by having large intracytoplasmic granules and an irregular outline; whereas, type B cells had a large amount of rough endoplasmic reticulum and a regular outline. In normal synovium, there was no apparent transition between cell types; however, in infected joints, changes in the lining cells were indicative of a gradual transition of type B cells into type A cells. Hypertrophic type B cells located in the subsurface layer of lining cells appeared to gradually change into polarized cells characteristic of type B cells at the base and of type A cells at the apex. Type A cells, containing large granules believed to be secretory product, then developed at the synovial surface. The composition of the granules is speculative; however, they may represent hyaluronateprotein. Structural evidence was found indicating release of cytoplasmic contents from type A cells by the formation and rupture of cytoplasmic protrusions. Whether this is a secretory or degenerative process is questionable. Numerous pinocytotic and micropinocytotic vesicles were found in lining cells, especially type A cells, indicating the uptake of some

extracellular substance; however, phagocytic vacuoles containing particulate matter were not found in lining cells of intact synovium. Most phagocytic cells in the synovial membrane and synovial fluid were believed to be monocytes; a few in the synovial fluid were morphologically similar to lining cells. Other ultrastructural changes complemented those seen with the light microscope.

Fluorescent antibody technique was the most satisfactory of several light-microscopic methods used to detect the OCP agent in McCoy cell cultures. However, in infected synovial tissues the results of fluorescent antibody staining were less satisfactory because of the small number of organisms present and confusing autofluorescence of eosinophils. Organisms were detected in infected cell cultures with Giemsa staining but could not be identified in synovial tissues because of inability to differentiate them from phagocytic debris. Electron microscopy revealed numerous organisms in infected cell cultures but very few in synovial tissues. Organisms in synovial tissues were either extracellular or within phagocytic cells where they apparently were being destroyed by lysosomal enzymes. Rapid destruction of organisms by such cells may account for the scarcity of organisms in synovial tissues.

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